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(54) Titre: GENES ET PROTEINES DE CASSETTE DE LIAISON AVEC ATP, DESTINES AU DIAGNOSTIC ET AU TRAITEMENT DE DESORDRES LIPIDIQUES ET MALADIES INFLAMMATOIRES

## (57) Abstract

Modulation of the activity of transmembrane proteins belonging to the ATP binding cassette (ABC) transporter protein family which are etiologically involved in cholesterol driven atherogenic processes and inflammatory diseases like psoriasis, lupus erythematoses and others provides therapeutic means to treat such diseases. Furthermore, detection of herein identified ABC transporter proteins of their respective biochemical activities involved in such atherogenic and inflammatory processes provides diagnostic means for clinical application of diagnosis and monitoring of dyslipidemias, atherosclerosis or inflammatory diseases like psoriasis and lupus erythematoses.

## (57) Abrégé

Selon l'invention, la modulation de l'activité de protéines transmembranaires qui appartiennent à la famille de protéines de transport (ABC) de cassette de liaison avec ATP et sont impliquées de manière étiologique dans des processus athérogènes provoqués par le cholestérol et dans des maladies inflammatoires comme le psoriasis, le lupus érythémateux et autres, constitue un moyen thérapeutique de traiter de telles maladies. En outre, la détection des protéines de transport (ABC) ici identifiées et de leurs activités biochimiques respectives, impliquées dans de tels processus athérogènes et inflammatoires, constitue un moyen de diagnostic destiné à l'application clinique de diagnostic et de surveillance des dyslipidémies, de l'athérosclérose ou de maladies inflammatoires telles que le psoriasis ou le lupus érythémateux.

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(54) Title: <b>ATP BINDING CASSETTE GENES AND PROTEINS FOR DIAGNOSIS AND TREATMENT OF LIPID DISORDERS AND INFLAMMATORY DISEASES</b>			
(57) Abstract <p>Modulation of the activity of transmembrane proteins belonging to the ATP binding cassette (ABC) transporter protein family which are etiologically involved in cholesterol driven atherogenic processes and inflammatory diseases like psoriasis, lupus erythematoses and others provides therapeutic means to treat such diseases. Furthermore, detection of herein identified ABC transporter proteins of their respective biochemical activities involved in such atherogenic and inflammatory processes provides diagnostic means for clinical application of diagnosis and monitoring of dyslipidemias, atherosclerosis or inflammatory diseases like psoriasis and lupus erythematoses.</p>			

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**Description**

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ATP binding cassette genes and proteins for diagnosis and treatment of lipid disorders and inflammatory diseases

Background of the invention

Reverse cholesterol transport mediated by HDL provides a "protective" mechanism for cell membrane integrity and foam cell formation and cellular cholesterol is taken up by circulating HDL or its precursor molecules. The precise mechanism of reverse cholesterol transport however is currently not fully understood and the mechanism of cellular cholesterol efflux and transfer from the cell surface to an acceptor-particle, such as HDL, is yet unclear. Certain candidate gene products have been postulated playing a role in the process of reverse cholesterol transport [1]. Apolipoproteins (e.g. ApoA-I, ApoA-IV), lipid transfer proteins (e.g. CETP, PLTP) and enzymes (e.g. LCAT, LPL, HL) are essential to exchange cholesterol and phospholipids in lipoprotein-lipoprotein and lipoprotein-cell interactions. Different plasma membrane receptors, such as SR-BI [2; 3], HB1/2 [4], and GPI-linked proteins (e.g. 120 kDa and 80 kDa) [5] as well as the sphingolipid rich microdomains (Caveolae, Rafts) of the plasma membrane have been implicated being involved in the process of reverse cholesterol transport and the exchange of phospholipids. How these membrane-microdomains are organized is in the current focus of interest for the identification of therapeutic targets. In recent studies SR-BI function as receptor for uptake of HDL into the liver and steroidogenic tissues could be demonstrated and the effectivity of this process is highly dependent on the phospholipid environment [2].

Cholesterol and phospholipid homeostasis in monocytes/macrophages and other cells involved in the atherosclerotic process is a critical determinant in atherosclerotic vessel disease. The phagocytic function of macrophages in host defense, tissue remodelling, uptake and lysosomal degradation of atherogenic lipoproteins and membrane fragments or other lipid containing particles has to be balanced by effective release mechanisms to avoid foam cell formation. HDL mediated reverse

cholesterol transport, supported by endogenous ApoE and CETP synthesis and secretion provides an effective mechanism to release excessive cholesterol from macrophages and other vascular cells.

Alternatively, reduced cholesterol and triglyceride/fatty acid absorption by intestinal mucosa cells as well as increased lipid secretion from hepatocytes into the bile will lower plasma lipids and the concentration of atherosclerotic lipoproteins.

#### Summary of the invention

New cholesterol responsive genes were identified with differential display method in human monocytes from peripheral blood that were subjected to macrophage differentiation and cholesterol loading with acetylated LDL and subsequent deloading with HDL<sub>3</sub>.

In an initial screen ABCG1 (ABC8), a member of the rapidly growing family of ABC (ATP-Binding Cassette) transport systems, that couple the energy of ATP hydrolysis to the translocation of solutes across biological membranes, was identified as a cholesterol sensitive switch. ABCG1 is upregulated by M-CSF dependent phagocytic differentiation but expression is massively induced by cholesterol loading and almost completely set back to differentiation dependent levels by HDL<sub>3</sub>.

In a more detailed analysis 37 already characterised ABC members and 8 Fragment - sequences (Table 2) were analysed in monocyte/macrophage cells by RT-PCR (linear range) for differentiation dependent changes and cholesterol sensitivity.

Among the 45 tested ABC-transporter genes 18 of the characterized ABC transporters and 2 of the Fragment -sequence based ABC-transporters are cholesterol sensitive (Example 4).

The cholesterol sensitive ABC-transporter are named according to the new ABC-

nomenclature and listed in Table 3 with the new and the old designations, respectively.

The most sensitive gene was ABCG1. ABCG1 is the human homologue of the drosophila white gene. Sequencing of the promoter of ABCG1 (Example 7) shows important transcription factor binding sites relevant for phagocytic differentiation and lipid sensitivity.

Antisense treatment of macrophages during cholesterol loading and HDL<sub>3</sub>-mediated deloading clearly identified ABCG1 as a cholesterol transporter and the efflux of choline-containing phospholipids (phosphatidylcholine, sphingomyelin) was also modulated. Northern- and Western-blot analysis provided further support that inhibition of cholesterol transport is associated with lower ABCG1 mRNA expression and ABCG1 protein levels (Example 5).

Considerable evidence was derived from energy transfer experiments (Example 3) that ABCG1 in the cell membrane is in a regulated functional cooperation (e.g. cell differentiation, activation, cholesterol loading and deloading) with other membrane receptors that have either transport- (e.g. LRP-LDL receptor related protein) or signalling- and adhesion-function (e.g. integrins, integrin associated proteins) which is also supported by sequence homology of extracellular domains as well as other parts of the ABCG1 sequence. For example the protein sequence of the region of the third extracellular loop of ABCG1, i.e. aminoacid residues 580 through 644, shares homology with fibronectin (aa 317-327), integrin $\beta$ 5 (aa 538-547), RAP (aa 119-127), LRP (aa 2874-2894), apoB-100 precursor (aa 4328-4369), glutathion-S-transferase (aa 54-78) and glucose transporter (aa 371-380). Sequence comparison of all cholesterol sensitive transporters indicates this as a general principle of ABC transporter function and regulation.

Among the other cholesterol sensitive genes ABCA1 (ABCI) was further characterized. ABCA1 was identified in the mouse as an IL-1beta transporter

involved also in apoptotic cell processing. We show here, by RT-PCR (Table 2) and confirmation by Northern analysis, based on the newly detected human ABCA1 cDNA sequence (Example 6), that ABCA1 follows the same regulation as ABCG1.

Moreover, the ABCA1-knockout mice (ABCA1<sup>-/-</sup>) show massively reduced levels of serum lipids and lipoproteins. The expression of ABCA1 in mucosa cells of the small intestine and the altered lipoprotein metabolism in ABCA1<sup>-/-</sup> mice allows the conclusion that ABCA1 plays a major role in intestinal absorption and translocation of lipids into the lymph-system

Analysis of genetic defects that affect macrophage cholesterol homeostasis identified dysregulated ABCA1 as a gene locus involved in the HDL-deficiency syndrome (Tangier-Disease). This disease is associated with hypertriglyceridemia and splenomegaly.

Another as yet not described HDL-deficiency syndrome associated with early onset of coronary heart disease and psoriasis showed a dysregulation of the chromosome 17 associated ABC-sequences (ABCC4 (MRP3); ABCC3 (MRP3); ABCA5 (Fragment 90625); ABCA6 (Fragment 155051) :17q21-24). This points to an association with the predicted gene locus for psoriasis at chromosome 17.

A recently sequenced human ABC-transporter (ABCA8, Example 9) shows high homology to ABCA1 and also belongs to the group of cholesterol sensitive ABC-transporter.

ABCC5 (MRP5, sMRP) is a member of the MRP-subfamily among which ABCC2 (MRP2, cMOAT) was characterized as the hepatocyte canalicular membrane transporter that is involved in bilirubin glucuronide secretion [9] and identified as the gene locus for Dubin-Johnson Syndrome [10] a disorder associated with mild chronic conjugated hyperbilirubinemia.



Furthermore, the identification of ABCA1 as a transporter for IL-1  $\beta$  identifies this gene as a candidate gene for treatment of inflammatory diseases including rheumatoid arthritis and septic shock. The cytokine IL-1  $\beta$  is a broadly acting proinflammatory mediator that has been implicated in the pathogenesis of these diseases.

Moreover, we could demonstrate, that glyburide as an inhibitor of IL-1  $\beta$  secretion inhibits not only Caspase I mediated processing of pro-IL-1  $\beta$  and release of mature IL-1  $\beta$  but simultaneously inhibits ceramide formation from sphingomyelin mediated by neutral sphingomyelinase and thereby releases human fibroblasts from G<sub>2</sub>-phase cell cycle arrest. These data provide a further mechanism indicative for a function of ABCA1 in signalling and cellular lipid metabolism.

Autoimmune disorders that are associated with the antiphospholipid syndrome (e.g. lupus erythematoses) can be related to dysregulation of B-cell and T-cell function, aberrant antigen processing, or aberrations in the asymmetric distribution of membrane phospholipids. ABC-transporters are, besides their transport function, candidate genes for phospholipid translocases, floppases and scramblases that regulate phospholipid asymmetry (outer leaflet: PC+SPM; inner leaflet: PS+PE) of biological membranes [11]. There is considerable evidence for a dysregulation of the analysed ABC-transporters in patient cells. We conclude that these ABC-cassettes are also candidate genes for a genetic basis of antiphospholipid syndromes such as in Lupus erythematoses.

In summary, the ABC genes ABCG1, ABCA1 and the other cholesterol-sensitive ABC genes as specified herein, can be used for diagnostic and therapeutic applications as well as for biochemical or cell-based assays to screen for pharmacologically active compounds which can be used for treatment of lipid disorders, atherosclerosis or other inflammatory diseases. Thus it is an objective of the present invention to provide assays to screen for pharmacologically active compounds which can be used for treatment of lipid disorders, atherosclerosis or

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other inflammatory diseases. Further the invention provides tools to identify modulators of these genes and gene products. These modulators can be used for the treatment of lipid disorders, atherosclerosis or other inflammatory diseases or for the the preparation of medicaments for treatment of lipid disorders, atherosclerosis or other inflammatory diseases. The medicaments comprise besides the modulator acceptable and usefull pharmaceutical carriers.

## Abbreviations

aa	Amino acid
ABC	ATP-binding cassette
ABCA#	ATP-binding cassette, sub-family A (ABC1), member #
ABCB#	ATP-binding cassette, sub-family B (MDR/TAP), member #
ABCC#	ATP-binding cassette, sub-family C (CFTR/MRP), member #
ABCD#	ATP-binding cassette, sub-family D (ALD), member #
ABCE#	ATP-binding cassette, sub-family E (OABP), member #
ABCF#	ATP-binding cassette, sub-family F (GCN20), member #
ABCG#	ATP-binding cassette, sub-family G (WHITE), member #
ABCR	Homo sapiens rim ABC transporter
AcLDL	Acetylated LDL
ADP1	ATP-dependent permease
ALDP	Adrenoleukodystrophy protein
ALDR	Adrenoleukodystrophy related protein
ApoA	Apolipoprotein A
ApoE	Apolipoprotein E
ARA	Anthracycline resistance associated protein
AS	Antisense
ATP	Adenosine triphosphate
CETP	Cholesteryl ester transfer protein
CFTR	Cystic fibrosis transmembrane conductance regulator
CGT	ceramide glucosyl transferase
CH	Cholesterol
cMOAT	Canalicular multispecific organic anion transporter
dsRNA	Double stranded RNA
Fragment	Gen Fragment
FABP	plasma membrane fatty acid binding protein

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FACS	Fluorescence activated cell sorter
FATP	intracellular fatty acid binding protein
FCS	foetal calve serum
FFA	free fatty acids
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCN20	protein kinase that phosphorylates the alpha-subunit of translation initiation factor 2
GPI	Glycosylphosphatidylinositol
HaCaT	keratinocytic cell line
HDL	High density lipoprotein
HL	Hepatic lipase
HlyB	haemolysin translocator protein B
HMT1	yeast heavy metal tolerance protein
HPTLC	High performance thin layer chromatography
IL	Interleukin
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LRP	LDL receptor related protein
MDR	Multidrug resistance
MRP	Multidrug resistance-associated protein
PC	Phosphatidylcholine
PE	Phosphatidylethanolamin
PL	Phospholipid
PLTP	Phospholipid transferprotein
PMP	peroxisomal membrane protein
PS	Phosphatidylserine
RNA	Ribonucleic acid
RT-PCR	Reverse transcription – polymerase chain reaction
SDS	Sodium dodecyl sulfate

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SL	Sphingolipid
sMRP	Small form of MRP
SPM	Sphingomyelin
SR-BI	Scavenger receptor BI
SUR	Sulfonylurea receptor
TAP	Antigen peptide transporter
TG	Triglycerides
TSAP	TNF-alpha stimulated ABC protein
UTR	untranslated region

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**Description of the Figures**

Figures 1 to 5 are showing nucleotide and protein sequences described in this application. The sequences are repeated in the sequence listing.

**Description of Tabela:****Table 1:**

Levels of RNA transcripts of ABCG1 (ABC8), ABCA1 (ABC1) and ABCA8 in human tissues were determined by Northern blot analysis of a multiple tissue dot-blot (Human RNA MasterBlot, Clontech Laboratories, Inc., CA, USA ). The relative amount of expression is indicated by different numbers of filled circles.

**Table 2:**

The expression pattern of ABC-transporters in monocytes, monocyte derived macrophages (3 days cultivated monocytes in serum free Macrophage-SFM medium containing 50 ng/ml M-CSF), AcLDL incubated monocytes (3 days with 100 µg/ml) followed by HDL<sub>3</sub> (100 µg/ml) incubated monocytes is shown. Expressed genes are tested for cholesterol sensitivity by semiquantitative PCR.

For known ABC-Transporter the chromosomal location and the transported molecules are also presented.

**Table 3:**

Disorders, that are associated with ABC-transporters are shown. The chromosomal location is indicated and the relevant accession number in OMIN (Online Mendelian Inheritance in Man).

**Table 4:**

Expression of ABC-Transporters in HaCaT keratinocytic cells during differentiation

Table 1

<i>Tissue</i>	ABCG1 (ABC8)	ABCA1 (ABC1)
Adrenal gland	•••••	••••
Thymus	••••	••
Lung	••••	••••
Heart	••••	••
Skeletal	••	•
Brain	••••	••
Spleen	•••••	••
Lymphnode	••••	•
Pancreas	•	•
Placenta	••••	•••••
Colon	••	•
Small intestine	••	••••
Prostate	••	•
Testis	•	•
Ovary	••	•
Uterus	•	••
Mammary gland	••	•
Thyroid gland	••	••
Kidney	••	•
Liver	••••	••••
Bone marrow	•	•
Peripheral leukocytes	•	•
<i>Fetal tissue</i>		
Fetal brain	•	••
Fetal liver	•	••••
Fetal spleen	••	••••
Fetal thymus	••	••
Fetal lung	••	••••

Table 2: Cholesterol dependent gene regulation of human ABC transporters

Gene	chromosomal localization	peripheral blood monocytes	3 days old M-CSF M $\phi$	cholesterol loading (acLDL)	cholesterol de-loading (HDL3)	transported molecules
ABCG1 (ABC8)	21q22.3	+	↑	↑↑	↓↓	cholesterol / choline PL
ABCA1 (ABC1)	9q22-31	+	↑	↑↑	↓↓	cholesterol / IL-10
ABCC5 (MRP5)	3q25-27	+	↑	↑↑	↓	
ABCD1 (ALDP, ALD)	Xq28	+	↑	↑	↓	very long chain fatty acids
ABCA5 (est90625)	17q21-25	+	↑	↑	↓	
ABCB11 (BSEP, SPGP)	2q24	+	↑	↑↑	↓	bile acids
ABCA8 (ABC-new)		+	+	↑	↓	
ABCC2 (MRP2)	10q23-24	+	+	↑	↓	bilirubin glucuronide
ABCB6 (est45597)	2q33-36	+	+	↑	↓	
ABCC1 (MRP1)	16p13.12	+	↓	↑	↓	eicosanoids
ABCA3 (ABC3)	16p13.3	+	↑	↑	nr	
est1133530		+	↑	↑	nr	
ABCB4 (MDR3)	7q21	+	↑	↓	↑	phosphatidylcholine
ABCG2 (est157481, ABCP)	4q22-23	+	↑	↓	↑	
ABCC4 (MRP4)	13q31	+	↑	↓	↑	
ABCB9 (est122234)	12q24	+	↑	↓	↑	
ABCD2 (ALDR)	12q11	+	↓	↓	↑	very long chain fatty acids
ABCB1 (MDR1)	7q21	+	+	↓	↑	phospholipids, amphiphiles
ABCA6 (est155051)	17q21	+	↑	↓	nr	
est640918		+	↑	↓	nr	
ABCD4 (P70R)	14q24.3	+	↑	nr	nr	
ABCA2 (ABC2)	9q34	+	↑	nr	nr	
ABCF2 (est133090)	7q35-36	+	↑	nr	nr	
ABCB7 (ABC7)	Xq13.1-3	+	↑	nr	nr	iron
ABCF1 (ABC50, TSAP)	6p21.33	+	↑	nr	nr	
ABCC6 (MRP6)	16p13.11	+	↓	nr	nr	
ABCB5 (est422562)	7p14	+	↓	nr	nr	
ABCC3 (MRP3)	17q11-21	+	nr	nr	nr	
ABCA4 (ABCR)	1p22	+	nr	nr	nr	retinoids, lipofuscin
ABCB2 (TAP1)	6p21.3	+	nr	nr	nr	peptides
ABCB3 (TAP2)	6p21.3	+	nr	nr	nr	peptides

Gene	chromosomal localization	peripheral blood monocytes	3 days old M-CSF M $\square$	cholesterol loading (acLDL)	cholesterol deloading (HDL3)	transported molecules
ABCF3 (est201864)	3q25.1-2	+	nr	nr	nr	
ABCB8 (est328128)	7q35-36	+	$\uparrow$	nr	nr	
ABCE1 (OABP)	4q31	+	$\uparrow$	nr	nr	
ABCB10 (est20237)	1q32	+	$\uparrow$	nr	nr	
est698739		+	$\uparrow$	nr	nr	
ABCC10 (est182763)	6p21	+	nr	nr	nr	
ABCC7 (CFTR)	7q31	$\emptyset$	$\emptyset$	$\emptyset$	$\emptyset$	ions
ABCC8 (SUR-1)	11p15.1	$\emptyset$	$\emptyset$	$\emptyset$	$\emptyset$	
ABCD3 (PMP70)	1p21-22	$\emptyset$	$\emptyset$	$\emptyset$	$\emptyset$	
Huwhite2		$\emptyset$	$\emptyset$	$\emptyset$	$\emptyset$	
est1125168		$\emptyset$	$\emptyset$	$\emptyset$	$\emptyset$	
est1203215		$\emptyset$	$\emptyset$	$\emptyset$	$\emptyset$	
est168043		$\emptyset$	$\emptyset$	$\emptyset$	$\emptyset$	
est990006		$\emptyset$	$\emptyset$	$\emptyset$	$\emptyset$	

+ = expressed

 $\emptyset$  = not expressed

nr=not regulated

 $\uparrow$  = upregulated $\downarrow$  = downregulated

half (hs) or full size (fs) transporter as deduced from the mRNA size

Table 3

<i>Disorders</i>	<i>Genomic location</i>	<i>Associated gene</i>	<i>OMIM-acc.nr.</i>
<b>Metabolic disorders:</b>			
Cystic fibrosis	7q31.3	ABCC7 (CFTR)	219700
Dubin Johnson syndrome (mild chronic conjugated hyperbilirubinemia)	10q24	ABCC2 (CMOAT)	237500
Progressive familial intrahepatic cholestasis type III (PFIC3)	7q21.1	ABCB4 (MDR3)	602347
Byler disease (PFIC2)	2q24	ABCB11 (BSEP, sPGP)	601847
Familial persistent hyperinsulinemic hypoglycemia	11p15.1	ABCC8 (SUR-1)	601820
IDDM	6p21.3	ABCB2 (TAP1)/ABCB3 (TAP2)	222100
<b>Neuronal disorders:</b>			
Adrenoleukodystrophy	12q11	ABCD2 (ALDR)	300100
Zellweger's syndrome	1p22-21	ABCD3 (PMP70)	214100
Multiple Sclerosis	6p21.3	ABCB2 (TAP1)/ABCB3 (TAP2)	126200
X-linked Sideroblastic anemia with spinocerebellar ataxia	Xq13.1-3	ABCB7 (ABC7)	301310
Menkes disease (altered homeostasis of metals)	Xq13	ABCB7 (ABC7)	309400
<b>Immune/Hemostats disorders:</b>			
Herpes simplex virus infection [12]	6p21.3	ABCB2 (TAP1)/ABCB3 (TAP2)	
Behcet's syndrome	6p21.3	ABCB2 (TAP1)/ABCB3 (TAP2)	109650
Bare lymphocyte syndrome type I	6p21.3	ABCB2 (TAP1)/ABCB3 (TAP2)	209920
Scott syndrome	7q21.1	ABCB1 (MDR1)	262890
<b>Retinal dystrophies:</b>			
Fundus flavi maculatus with macular dystrophy	1p13-21	ABCA4 (ABCR)	601691
Juvenile Stargardt disease	1p13-21	ABCA4 (ABCR)	248200
Age-related macular degeneration	1p13-21	ABCA4 (ABCR)	153800
Cone-rod dystrophy	1p13-21	ABCA4 (ABCR)	600110
Retinitis pigmentosa	1p13-21	ABCA4 (ABCR)	601718

<i>Diseases with evidence for involvement of ATPcassettes/translocases and floppases[80]</i>		<i>Assumed gene</i>	
BRIC (Benign recurrent intrahepatic obstructive jaundice)	18	Assumed	243300
Psoriasis	17q11-12 17q21-24	ABCA5 (Fragment 90625) ABCC3 (MRP3)	602723 177900 601454
Lupus erythematoses -- Antiphospholipid Syndrome		Translocase Flippase	152700
PFIC(Prog. Fatal familial intrahepatic choestasis) PFIC1	18q21-22	ATP Transporters	211600
<i>Neurological disorders mapped to gene locus of ABCG1 (ABC8)</i>			
Autosomal bipolar affective disorder	21q22.3	ABCG1 (ABC8)	125480
Autosomal recessive non-syndromic deafness	21q22.3	ABCG1 (ABC8)	601072
Down Syndrome (ABC-8 may be a candidate for the Brushfield spots -- mottled, marble or speckled irides frequently seen in Down- Syndrome)	21q22.3	ABCG1 (ABC8)	190685
Linkage to phosphofructokinase (liver type)	21q22		171860
<i>HDL-deficiency syndromes.</i> Gen responsible for Tangier Disease	9q31	ABCA1 (ABC1)	205400

Table 4: Expression of ABC-Transporters in HaCaT keratinocytic cells during differentiation

Gene	chrom. localisation	initial expression	differentiation dependent expression	known or putative molecules transported
ABCG1 (ABC8)	21 q22.3	+++++	↑	cholesterol choline-PL
ABCC3 (MRP3)	17 q11-q12	++++	↑	
ABCA8	19 p13	+++++	↑	
ABCC1 (MRP1)	16 p13	+++++	↗ ↘ (max. day 2)	PGA <sub>1</sub> , LTC <sub>4</sub> DNP-SG
ABCD4 (PMP69, P70R)	14 q24	+++++	↗ ↘ (max. day 2,4)	
ABCC2 (MRP2)	10 q24	+++	↗ ↘ (max. day 2)	bilirubin glucuronide
ABCA3 (ABC3)	16 p13	+	↗ ↘ (max. day 4,6)	
ABCA5 (ABCR)	1 p21	+	↗ ↘ (max. day 4)	retinoid, lipofuscin
ABCA1 (ABC1)	9 q22-q31	+	↗ ↘ (max. day 6)	
ABCC6 (MRP6)	16 p13.11	+	↗ ↘ (max. day 4)	
ABCC4 (MRP4)	13 q31	++++	↗ ↘ (max. day 2,4)	
ABCA2	9 q34	++++	↗ ↘ (max. day 6)	
ABCC5 (MRP5, SMRP)	3 q27	+++++	↗ ↘ (max. day 2,4)	



5	ABCB6 (est45597)	2	++++	↗ ↘ (max. day 2,4)	
10	ABCB7 (ABC7)	X q13.1-3	++++	↗ ↘ (max. day 4)	irons
	TAP1 (ABCB1)	6 p21.3	++++	↗ ↘ (max. day 4,6)	peptides
	TAP2 (ABCB2)	6 p21.3	++++	↗ ↘ (max. day 2,4)	peptides
15	ABCB8 (est328128)	7 q35-36	++++	↗ ↘ (max day 2)	
	EST640918	17 q24	+	↗ ↘ (max day 4)	
20	ABCC7 (CFTR)	7 q31	+++	↗ ↘ (max day 4)	
	ABCB10 (est20237)	1 q32	+++	↗ ↘ (max. day 2)	
25	ABCF1 (TSAP)	6 p21.33	++++	↓	
	ABCC10 (est182763)	1 q32	++++	↓	
	ABCE1 (OABP)	4 q31	++++	↓	
30	EST698739	17 q24	++++	↓	
	ABCF2 (est133090)	7 q35-q36	++++	↓	
35	ALD (ABCD1,ALDP)	X q28	++++	↓	VLCFA
	ABCA5 (est90625)	17 q21-q24	+++	↓	
	ABCB5 (est422562)	7 p14	++++	↓	
40	ABCB9 (est122234)	12 q24-qter	++	↓	
	ABCD2 (ALDR)	12 q11	+	↓	VLCFA
45	ABCF3 (est201864)	3 q25.1-2	++++	↓	
	ABCG2 (ABCI5,ABCP)	4 q22-q23	++++	↓	
50	EST1133530	4 p16pter	++++	↓	

Ilwwhite	11 q23	++++	↓	
ABCA6 (cst155051)	17 q21	++	↓	
BSEP (ABCB11, sPGP)	2 q24	+	↓↑ (max day 6)	
ABCB4 (MDR3)	7 q21	not expressed		phosphatidyl- choline
ABCD3 (PMP70)	1 p22	not expressed		
ABCB1 (MDR1)	7 q21	not expressed		phospholipids amphiphiles
EST168043	2 p15-16	not expressed		
EST990006	17 q24	not expressed		
ABCC8 (SUR1)	11 p15.1	not expressed		

+: relative expression    n.d.: not determined

↑: upregulated    ↓: downregulated    ↗↘: biphasic expression

### Description of specific embodiments

#### **Candidate gene identification during cholesterol loading and deloading of human monocyte derived macrophages**

In order to discover genes that are involved in the cholesterol loading and/or deloading in vitro assays were set up. Particularly, gene expression in human blood derived monocytes and macrophages elicited by cholesterol and its physiological transport formulation, i.e. various low density lipoprotein (LDL) particle species like AcLDL, was studied.

Elutriated human monocytes were cultivated in M-CSF containing but serum free macrophage medium supplemented with AcLDL (100 µg protein/ml medium) for three days, followed by cholesterol depletion replacing AcLDL by HDL<sub>3</sub> (100 µg protein/ml medium) for twelve hours. Differential display screening for new candidate genes, regulated by cholesterol loading/deloading, was performed (Example 1).

#### **Identification of a new cholesterol sensitive gene**

ABCG1 (ABC8) was discovered as a novel cholesterol sensitive gene. ABCG1 belongs to the ATP binding cassette (ABC) transporter gene family. ABCG1 was recently published as the human analogue of the drosophila white gene [6-8].

The gene is strongly upregulated by AcLDL-mediated cholesterol loading, and almost completely downregulated by HDL<sub>3</sub> mediated-cholesterol deloading, as confirmed by Northern blot (Example 2). Northern blot analysis of mRNA from human monocyte-derived macrophages obtained from the peripheral blood probands clearly show upregulation of ABCG1 mRNA formation upon AcLDL incubation. In sharp contrast, ABCG1 mRNA expression was decreased in such macrophages upon incubation with HDL<sub>3</sub> containing medium.

### ABCG1 expression in cholesterol loaded and deloaded cells after four days pre-differentiation

For effective cholesterol loading monocytes must be differentiated to phagocytic-macrophage like cells. During this period scavenger receptors are upregulated and promote AcLDL uptake leading to cholesteryl ester accumulation. After four days preincubation period we have incubated the cells for one, two and three days with AcLDL (100 µg/ml) to show cholesteryl ester accumulation. After two days of loading we deloaded the cells with HDL<sub>3</sub> for 12 hours, 24 hours and 48 hours, respectively. ABCG1 is time dependently upregulated during the AcLDL loading period and downregulated by HDL<sub>3</sub> deloading (Examples 2 and 3) In order to confirm time dependent increase of ABCG1 mRNA expression after AcLDL challenge in human monocyte derived macrophages, Northern blot analyses for ABCG1 mRNA quantification were made, RNA samples from the macrophages were harvested at day zero and day four as controls and mRNA samples were taken one, two, and three days after AcLDL treatment of macrophages, which started at day four. A dramatic increase of ABCG1 mRNA content of the macrophages could be detected from day five through day seven by Northern blot analyses.

This regulation shows the same pattern as changes of cellular cholesteryl ester content (Example 3). Cholesterol ester accumulation starts in monocyte-derived macrophages upon AcLDL stimulation from a base level below 5 nmol/mg cell protein at day four up to 120 nmol/mg cell protein at day seven (i.e. three days after AcLDL application).

### Tissue expression

Besides cholesterol loaded macrophages ABCG1 is prominently expressed in brain, spleen, lung, placenta, adrenal gland, thymus and fetal tissues (Table 1).

### Chromosomal location and associated genes and diseases

The ABCG1 gene maps to human chromosome 21q 22.3. Also localized in this region 21q 22.3 are the following genes: integrin  $\beta$  2 (CD18), brain specific polypeptide 19, down syndrome cell adhesion molecule, dsRNA specific adenosine deaminase, cystathionine  $\beta$  synthase, collagen VI alpha-2, collagen XVIII alpha-1, autosomal recessive deafness, and amyloid beta precursor.

This chromosomal region is in close proximity to other regions involved in Down syndrome, autosomal dominant bipolar affective disorder, and autosomal recessive non-syndromic deafness.

### Extracellular loop of ABCG1 (ABC8) for antibody generation

The putative structure of the hydrophobic transmembrane region of ABCG1 shows 6 transmembrane spanning domains, and 3 extracellular loops, two of them are 9- and 8-amino acids-long, respectively, while the third one is 66-amino acids-long.

The larger one of the two intracellular loops consists of 30 amino acids. Similarity-survey in protein databases for homologies the 3rd extracellular loop (IIIex) with other genes resulted in the identification of fibronectin, integrin $\beta$ 5, RAP, LRP (LDL receptor related protein) apo-lipoprotein B 100 precursor protein, glutathion S-transferase and glucose transporter.

A polyclonal antiserum was generated against the 3rd extracellular loop (IIIex) of ABCG1 in order to perform flow cytometric analysis, energy transfer experiments and Western-blotting (see Example 3). In the amino acid sequence of ABCG1 the 3rd extracellular loop (IIIex) comprises 66 amino acids from amino acid 580 through 644. The peptide fragment for antibody generation comprises the amino acid residues 613 through 628 of ABCG1 polypeptide. ABCG1 obviously interacts with endogenous sequence motifs with other membrane receptors

involved in transport (e.g. LRP, RAP), signalling and adhesion (e.g. integrins, integrin associated proteins) as a basis of ABCG1-function and regulation. Moreover sequence comparisons of all ABC-transporters listed in Table 3 indicates functional cooperation with other membrane receptors as a general principle of the whole gene family.

#### Subfamily-Analysis

Evolutionary relationship studies with the whole ABC transporter family have shown that ABCG1 (ABC8) forms a subfamily together ABCG2 (cst157481) and this subfamily is closely related to the full-size transporters ABCA1 (ABC1), ABCA2 (ABC2), ABCA3 (ABC3), ABCA4 (ABCR) and the half-size transporter ABCF1 (TSAP).

Recent studies by Allikmets et al. have identified 21 new genes as ABC transporters by expressed sequence tags database search [13].

#### General description of the ABC transporter family

The ATP-binding cassette (ABC) transporter superfamily contains some of the most functionally diverse proteins known. Most of the members of the ABC family (also called traffic ATP-ases) function as ATP-dependent active transporters (Table 3). The typical functional unit consists of a pair of ATP-binding domains and a set of transmembrane (TM) domains. The TM-domains determine the specificity for the type of molecule transported, and the ATP-binding domains provide the energy to move the molecule through the membrane [14; 15]. The variety of substrates handled by different ABC-transporters is enormous and ranges from ions to peptides. Specific transporters are found for nutrients, endogenous toxins, xenobiotics, peptides, aminoacids, sugars, organic/inorganic ions, vitamins, steroid hormones and drugs [16; 17].

### ABC-transporter associated diseases

The search for human disease genes (Table 3) provided a number of previously undiscovered ABC proteins [16]. The best characterized disease caused by a mutation in an ABC transporter is cystic fibrosis (ABCC7 (CFTR)). Inherited disorders of peroxisomal metabolism as Adrenoleukodystrophy and Zellweger's syndrome also show alterations in ABC transporters. They are involved in peroxisomal beta-oxidation, necessary for very long chain fatty acid metabolism [18].

### Antisense against ABCG1 inhibits cholesterol efflux to HDL<sub>3</sub>

Since ABCG1 is a cholesterol sensitive gene and other ABC transporters are known to be involved in certain lipid transport processes, the question arises whether ABCG1 plays a role in transport of cholesterol, phospholipids, fatty acids or glycerols. Therefore antisense experiments were performed to test the influence of ABCG1 on lipid loading and deloading. The inhibition of ABCG1 with specific antisense oligonucleotides decreased the efflux of cholesterol and phosphatidylcholine to HDL<sub>3</sub>. (Example 5)

### Other cholesterol sensitive ABC transporter

Cloning and sequencing of the human ABCA1 (ABC1) provided the information to characterize ABCA1 for cholesterol sensitivity, and tissue distribution (Example 6). Another cholesterol sensitive human ABC transporter (ABCA8) has been cloned and sequenced (Example 8)

### Characterization of the ABCG1 promoter region

The ABCG1 promoter has the characteristic binding sites for transcription factors that are involved in the differentiation of monocytes into phagocytic macrophages. The cholesterol sensitivity of the expression of ABCG1 is represented by the transcription factor pattern that is relevant for phagocytic differentiation (Example 7).

## Examples

### Example 1

#### 5 Identification of cholesterol loading and deloading candidate genes

##### 15 Monocyte isolation and cell culture

Monocytes were obtained from peripheral blood of healthy normolipidemic volunteers by leukapheresis and purified by counterflow elutriation. Purity of isolated monocytes was >95% as revealed by FACS analysis.  $10 \times 10^6$  monocytes were seeded into 100 mm<sup>2</sup> diameters cell culture dishes under serum free conditions in macrophage medium for 12 hours in a humidified 37°C incubator maintained with a 5% CO<sub>2</sub>, 95% air atmosphere. After 12 hours medium containing unattached cells was replaced by fresh macrophage medium supplemented with 50 ng/ml human recombinant M-CSF (this medium is the standard medium for any further incubations).

##### 30 Isolation of lipoproteins and preparation of AcLDL

Lipoproteins were prepared from human plasma from healthy volunteer donors by standard sequential ultracentrifugation methods in a Beckman L-70 ultracentrifuge equipped with a 70 Ti rotor at 4°C to obtain LDL ( $d=1,006$  to  $1,063$  g/ml) and HDL<sub>3</sub> ( $d=1,125$  to  $1,21$  g/ml). All densities were adjusted with solid KBr. Lipoprotein fractions are extensively dialyzed with phosphate-buffered saline (PBS) containing 5 mM EDTA. The final dialysis step was in 0,15 mol/L NaCl in the absence of EDTA. Lipoproteins were made sterile by filtration through a 0.45 µm (pore-size) sterile filter (Sartorius).

LDL was acetylated by repeated addition of acetic anhydride followed by dialysis against PBS [19]. Modified LDL showed enhanced mobility on agarose gel electrophoresis.



#### Incubation of monocyte-macrophages with AcLDL and HDL<sub>3</sub>

After 12 hours of preincubation cells were grown in the presence or absence (control) of 100 µg protein /ml AcLDL for further 3 day in medium. Then, the incubation medium was replaced with fresh medium and incubated with or without the addition of HDL<sub>3</sub> (100 µg/ml) for another 12 hours.

#### Differential display

Differential display screening was performed for new candidate genes that are regulated by cholesterol loading/deloading as described [20; 21]. In brief, 0,2 µg of total RNA isolated from monocytes at various incubations was reverse transcribed with specific anchored oligo-dT primers, using a commercially available kit (GeneAmp RNA PCR Core Kit, Perkin Elmer, Germany). The oligo-dT primers used had two additional nucleotides at their 3' end consisting of an invariable A at the second last position (3'-end) and A, C, G or T at the last position to allow a subset of mRNAs to be reverse transcribed. Here, a 13-mer oligo-dT (T101: 5'T11AG-2' ) was used in a 20-µl reaction at 2,5 µM concentration. One tenth of the cDNA was amplified in a 20-µl PCR reaction using the same oligo-dT and an arbitrary 10-mer upstream primer (D20 5'-GATCAATCGC-3'), 2,5 µM each, using 2,5 units of TAQ DNA Polymerase and 1.25 mM MgCl<sub>2</sub>. Amplification was for 40 cycles with denaturation at 94°C for 30 sec, annealing at 41°C for 1 min and elongation at 72°C for 30 sec with a 5 min extension at 72°C following the last cycle. All PCR reactions were carried out in a Perkin Elmer 9600 thermocycler (Perkin Elmer, Germany). PCR-products were separated on ready to use 10% polyacrylamide gels with a 5% stacking gel (CleanGel Large-10/40 ETC, Germany) under non-denaturing conditions using the Multiphor II electrophoresis apparatus (Pharmacia, Germany). The DNA fragments were visualized by silverstaining of the gel as previously described [22].

#### Cloning and sequencing of differentially expressed cDNAs

cDNA bands of interest were cut out of the gel and DNA was isolated by boiling the gel slice for 10 min in 20 µl of water. A 4 µl aliquot was used for the following PCR-reaction in a 20µl volume. The cDNA was reamplified using the same primer set and PCR conditions as above, except, that the final dNTP concentration was 1mM each. Reamplified cDNAs were cloned in the pUC18-vector using ABCC8 (SUR)eClone-Kit (Pharmacia), sequenced on an automated fluorescence DNA sequencer using the AutoRead Sequencing Kit (Pharmacia, Germany) and used as probes for Northern blot analysis [23].

#### Example 2

##### Northern Blot analyses of monocytes and macrophages after 3 days AcLDL incubation followed by 12 hours HDL<sub>3</sub> incubation

Elutriated monocytes were incubated with AcLDL (100 µg/ml medium) for 2.5 days or differentiated for the same time without the addition of AcLDL as control. ABCG1 (ABC8) expression is 4 times stronger upregulated with AcLDL incubation than in differentiated monocytes. After the AcLDL incubation period cells were washed and incubated with HDL<sub>3</sub> for the next 12 hours or with medium alone as control. ABCG1 expression is almost completely downregulated by HDL<sub>3</sub> incubation and only moderately decreased in control incubation as confirmed by Northern blot. For effective cholesterol loading monocytes must be differentiated to macrophage like cells. During this period scavenger receptors are upregulated and promote AcLDL uptake leading to cholesteryl ester accumulation. To differentiated the cells prior to AcLDL-dependent cholesterol loading, we cultured the cells for four days in standard medium. At day four, cells were washed and incubated with AcLDL (100µg/ml medium) or in the absence of AcLDL as control for further one, two and three days to load the cells with cholesterol. At each timepoint cells were lysed with 0.1 % SDS and lipid was extracted as described in materials and methods and cellular cholesteryl ester was determined by HPTLC-separation. Cells were loaded time

5 dependently up to 120 nmol/mg cell protein after 3 days AcLDL loading, whereas in  
unloaded cells no cholesteryl ester accumulation could be observed.

10 To distinguish HDL<sub>3</sub> dependent and independent cholesterol efflux cells were pulsed  
5 with AcLDL (100 µg/ml) for three days with the coincubation of <sup>14</sup>C-cholesterol (1,5  
µCi/ml medium). Cells were washed and deloaded with HDL<sub>3</sub> (100 µg/ml) for 12  
15 hours, 24 hours and 48 hours, respectively. Cells were incubated without the addition  
of exogenous lipid-acceptors as a control. After chase period the content of <sup>14</sup>C-  
cholesterol was determined in the medium and in the cells by liquid scintillation as  
20 described in material and methods. The efflux of cholesterol is expressed in percent  
of cellular DPMs of total DPMs (counts in the cells plus medium) With HDL<sub>3</sub> the  
efflux is faster and more intense. than the efflux without the addition of HDL<sub>3</sub> as an  
25 endogenous lipid acceptor. After 12 hours cellular cholesterol content was reduced to  
68 % with HDL<sub>3</sub>-dependent deloading, and 86 % in HDL<sub>3</sub>-independent deloading.  
15 After 48 hours only 35 % of loaded <sup>14</sup>C-cholesterol was observed in the cells treated  
with HDL<sub>3</sub>. In contrast, 70 % of loaded <sup>14</sup>C-cholesterol was found in untreated cells

30 In AcLDL pulsed cells the RNA-expression of ABCG1 is upregulated whereas no  
upregulation appears in the cells that were not loaded with AcLDL. Cells that were  
20 loaded for two days with AcLDL were deloaded with HDL<sub>3</sub> for 12, 24 and 48 hours  
35 (12h; 24h; 48h), and in the absence of exogenous lipid acceptors. The RNA-  
expression is downregulated again, in HDL<sub>3</sub> treated cells more intense than in cells  
treated without any exogenous lipid acceptor.

#### 40 25 **Materials:**

Macrophage medium (Macrophage-SFM) was obtained from Gibco Life Techno-  
45 logies, Germany. Human recombinant M-CSF was obtained from Genzyme Dia-  
gnostics, Germany, and antisense phosphorothioate oligonucleotides were supplied  
by Biognostics, Germany. All other chemicals were purchased from Sigma. Nylon  
30 membranes and a32P-dCTP were obtained from Amersham, Germany, <sup>14</sup>C-

cholesterol and 3H-choline chloride from NEN, Germany, and cell culture dishes are Becton Dickinson, Germany

#### Isolation of total RNA and northern blotting

Total RNA was isolated at each time-point, before and after AcLDL incubation, and after HDL<sub>3</sub> incubation, respectively. Washed cells were solubilized in guanidine isothiocyanate followed by sedimentation of the extract through cesium chloride [24]. For Northern analysis, 10 µg/lane of total RNA samples were fractionated by electrophoresis in 1,2% agarose agarose gel containing 6% formaldehyde and blotted onto nylon membranes (Schleicher & Schüll, Germany). After crosslinking with UV-irradiation (Stratalinker model 1800, Stratagene, USA), the membranes were hybridized with a cDNA probe for ABCG1 (ABC8). Hybridization and washing conditions were performed as recommended by the manufacturer of the membrane.

#### Example 3

##### Westernblot analysis of monocytes and macrophages after cholesterol loading and deloading

Protein expression of ABCG1 (ABC8) is upregulated in AcLDL-loaded and down-regulated in HDL<sub>3</sub>-deloaded monocyte-derived macrophages. Western blotting with a peptide antibody against ABCG1 as described in materials and methods is performed with 40 µg of total protein for each lane of SDS-PAGE. ABCG1-protein expression is shown in freshly isolated monocytes (day zero) and in differentiated monocytes (day four). From day four to day seven (5d; 6d; 7d) monocyte-derived macrophages were loaded with AcLDL or without AcLDL as control. AcLDL loaded cells from day 6 (6d) were deloaded with HDL<sub>3</sub> for 12, 24, and 48 hours and without exogenous added HDL lipid-acceptor. AcLDL increases the protein-expression, whereas HDL<sub>3</sub> decreases the expression to normal levels again.

#### Protein isolation and determination

At each timepoint cells were lysed with 0.1% SDS and the protein content was determined by the method of Lowry et al. [25].

#### Generation of ABCG1 specific antibodies

ABCG1 specific peptide antibodies were generated by immunization of chickens and rabbits with a synthetic peptide (Fa. Pineda, Berlin). The peptide sequence was chosen from the extracellular domain exIII amino acid residues 613-628 of ABCG1 comprising the amino acids REDLHCDIDETCHFQ (see sequence listing ID No. 53). After 58 days of immunization western blotting was performed with 1:1000 diluted serum and 1:10000 secondary peroxidase labelled antibody.

#### Electrophoresis and immunoblotting

SDS-polyacrylamide gelelectrophoresis was performed with 40µg total cellular protein per lane. Proteins were transferred to Immobilon as reported. Transfer was confirmed by Coomassie Blue staining of the gel after the electroblot. After blocking for at least 2 hours in 5% nonfat dry milk the blot was washed 3 times for 15 minutes in PBS. Antiserum generated as described was used at 1:1000 dilution in 5% nonfat dry milk in PBS. The blot was incubated for 1 hour. After 4 times washing with PBS at room-temperature a secondary peroxidase-labelled rabbit anti chicken IgG-antibody (1:10000 diluted, Sigma) was incubated in 5% nonfat dry milk in PBS for 1 hour. After 2 times washing with PBS, detection of the immune complexes was carried out with the ECL Western blot detection system (Amersham International PLC, UK).

#### Fluorescence resonance energy transfer:

Monocytes were labelled with the specific antibodies for 15 minutes on ice, one antibody is labelled by biotin, the other one is labelled by phycocrythrin. After washing the cells were incubated with a Cy5-conjugated streptavidin for another 15 minutes.

Distances between antibody labelled proteins on the cell surface is measured by energy transfer with a FACScan (Becton Dickinson). Following single laser excitation at 488 nm the Cy5 specific emission represents an indirect excitation of Cy5 dependent on the proximity of the PE-conjugated antibody. The relative transfer efficiency was calculated following standardisation for the intensity of PE and Cy5 labelling and nonspecific overlap of fluorescence based on dual laser excitation and comparison to separately stained control samples.

#### Example 4

##### Cholesterol sensitivity of ABCG1 (ABC8) and other members of the ABC-transporter family

The influence of cholesterol loading and deloading on other members of the ABC-family was also investigated to find out the potential second half-size ABC transporter.

Further analysis has been performed to examine the expression pattern of all human ABC transporters in monocytes and monocyte derived macrophages as well as in cholesterol loaden and deloaden mononuclear phagocytes.

The experiments were performed by RT-PCR with cycle-variation to compare the expression in the quantitative part of the distinct PCR. Primer sets were generated from the published sequences of the ABC-transporters. A RT-PCR with GAPDH primers was used as control.

Several ABC-transporters are also cholesterol sensitive which further supports the function of ABC-transporters in cellular lipid trafficking (Table 2).

##### Semi-quantitative RT-PCR

All known ABC-transporters are tested for AcLDL/HDL<sub>3</sub> sensitive regulation of expression using RT-PCR with cycle-variation to compare the expression in the

quantitative part of the distinct PCR. 1 µg of total RNA was used in a 40 µl reverse transcription reaction, using the Reverse Transkription System (Promega, Corp. WI, USA). Aliquots of 5 µl of this RT-reaction was used in 50µl PCR reaction. After denaturing for 1,5 min at 94°C, 35 or less cycles of PCR were performed with 92,3°C for 44s, 60,8°C for 40s (standard annealing temperature differs in certain primer-combinations), 71,5°C for 46s followed by a final 5-min extension at 72°C. The Primer sets were generated from the published sequences of the ABC-transporters. A RT-PCR with primers specific for GAPDH was performed as control.

The expression pattern of ABC-transporters in monocytes, monocyte derived macrophages (3 days cultivated monocytes in serum free macrophage-SFM medium containing 50 ng/ml M-CSF), AcLDL incubated monocytes (3 days with 100 µg/ml) followed by HDL<sub>3</sub> (100 µg/ml) incubated monocytes is shown in Table 2. Expressed genes are tested for cholesterol sensitivity by semi-quantitative PCR.

**Example 5:**

**Functional analyses of the cholesterol sensitive ABCG1 (ABC8) transporter gene by antisense oligonucleotide experiments**

Antisense experiments were conducted in order to address the question, that beyond being regulated by cholesterol loading and deloading ABCG1 is directly involved in lipid loading and deloading processes.

In various experiments antisense oligonucleotides decreased the efflux of cholesterol and phosphatidylcholine to HDL<sub>3</sub>. During the loading period with AcLDL the cells were coincubated with 17 different antisense oligonucleotides. To measure the efflux of cholesterol and phospholipids the cells were pulsed in the loading period with 1,5 µCi/ml <sup>14</sup>C-cholesterol and 3µCi/ml <sup>3</sup>H-choline chloride. The medium was changed and during the chase period cells were incubated with or without HDL<sub>3</sub> for 12 hours. The <sup>14</sup>C-cholesterol and <sup>3</sup>H-choline content in the medium and in the cell lysate was measured and the efflux was determined in percent of total <sup>14</sup>C-cholesterol and <sup>3</sup>H-choline loading.

5  
10 The most effective antisense oligonucleotide (AS Nr.2) inhibited cholesterol and phospholipids efflux relative to cells that were treated with control antisense (AS control). A dose dependent decrease in cholesterol efflux of 16,79% (5nmol AS) and 32,01% (10 nmol AS) could be shown, respectively.

15 **Antisense incubation**

To inhibit the induction of ABCG1 cells were treated with three different antisense oligonucleotides targeting ABCG1 or one scrambled control-antisense oligonucleotide during the AcLDL-incubation period.

20 **Determination of cholesterol and phosphatidylcholine efflux from monocytes in dependency of antisense oligonucleotide treatment**

25 To measure the efflux of cholesterol and phospholipids the cells were pulsed in addition to AcLDL-incubation with 1,5 µCi/ml <sup>14</sup>C-cholesterol and 3µCi/ml <sup>3</sup>H-choline chloride. The medium was changed and in chase period the cells were incubated with or without HDL<sub>3</sub> for 12 hours. Lipid extraction was performed  
30 according to the method of Bligh and Dyer [26]. The <sup>14</sup>C-cholesterol and <sup>3</sup>H-choline content in the medium and in the cell lysate was measured by liquid scintillation counting and the efflux was determined in percent of total <sup>14</sup>C-cholesterol and <sup>3</sup>H-choline loading as described [27]

35 **Computer analyses**

40 20 DNA and protein sequence analyses were conducted using programs provided by HUSAR, Heidelberg, Germany: <http://genius.embnet.dkfz-heidelberg.de:8080>.



### Example 6

Complete cDNA sequence of the human ATP binding cassette transporter 1 (ABCA1 (ABC1)) and assessing the cholesterol sensitive regulation of ABCA1 mRNA expression

#### cDNA Cloning and Primary Protein Structure

We have cloned a 6880-bp cDNA containing the complete coding region of the human ABCA1 gene (Figure 8) The open reading frame of 6603 bp encodes a 2201-amino acid protein with a predicted molecular weight of 220 kDa. This protein displays a 94% identity on the amino acid level in an alignment with mouse ABCA1 and can therefore be considered as the human ortholog.

#### Tissue Distribution of ABCA1 mRNA Expression

In order to examine the tissue-specific expression of ABCA1 a multiple tissue RNA master blot containing poly A<sup>+</sup> RNA from 50 human tissues was carried out. Northern Blot analysis demonstrates the presence of a ABCA1 specific signal in all tissues. It is mostly prominent in adrenal gland, liver, lung, placenta and all fetal tissues examined so far (Table 1). The weakest signals are found in kidney, pancreas, pituitary gland, mammary gland and bone marrow.

#### Sterol Regulation of ABCA1 mRNA Expression

In order to determine the regulation of ABCA1 in monocytes/macrophages during cholesterol loading/depletion Northern Blot analysis was performed. The cloned 1000-bp DNA fragment derived from PCR amplification of RNA from five day differentiated monocytes with primers ABCA1 3622f (CGTCAGCACTCTGATGATGGCCTG-3') and ABCA1 4620r (TCTCTGCTATCTCCAACCTCA-3') was hybridized to Northern Blots containing RNA of differentially cultivated monocytes (figure 12) As can be seen in lanes one to five, the ABCA1 mRNA is increased during in vitro differentiation of freshly isolated monocytes until day five. Longer cultivation results in a total loss of

expression. When the cells were incubated in the presence of AcLDL to induce sterol loading (lanes 6-8) beginning at day four, a much stronger accumulation of mRNA can be detected in comparison to control cells (lanes 2-5). When these cells were cultured with HDL<sub>3</sub> as cholesterol acceptor for 12h, 24h and 48h (lanes 9-11) the ABCA1 signal significantly decreases with respect to control cells incubated in the absence of HDL<sub>3</sub> (lanes 12-14). Taken together, these results indicate that ABCA1 is a sterol-sensitive gene which is induced by cholesterol loading and downregulated by cholesterol depletion.

#### Cell culture.

Peripheral blood monocytes were isolated by leukapheresis and counterflow elutriation (19JBC). To obtain fractions containing >90% CD 14 positive mononuclear phagocytes, cells were pooled and cultured on plastic Petri dishes in macrophage SFM medium (Gibco BRL) containing 25 U/ml recombinant human M-CSF (Genzyme) for various times in 5% CO<sub>2</sub> in air at 37°C. The cells were incubated in the absence (differentiation control) or presence of AcLDL (100 µg/ml) to induce sterol loading. Following this incubation the cells were cultured in fresh medium supplemented with or without HDL<sub>3</sub> (100 µg/ml) for additional times in order to achieve cholesterol efflux from the cells to its acceptor HDL<sub>3</sub>.

#### Preparation of RNA and Northern blot analysis.

Total cellular RNA was isolated from the cells by guanidium isothiocyanate lysis and CsCl centrifugation (Chirgwin). The RNA isolated was quantitated spectrophotometrically and 15 µg samples were separated on a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane (Schleicher & Schüll). After crosslinking with UV-irradiation (Stratalinker model 1800, Stratagene), the membranes were hybridized with a 1000 bp DNA fragment derived from PCR amplification with primers ABCA1 3622f and ABCA1 4620r, stripped and subsequently hybridized with a human β-actin probe. In order to determine the tissue-specific expression of ABCA1 a multiple tissue RNA master blot containing

poly A<sup>+</sup> RNA from 50 human tissues was purchased from Clontech. The probes were radiolabeled with [ $\gamma$ -<sup>32</sup>P]dCTP (Amersham) using the Oligolabeling kit from Pharmacia. Hybridization and washing conditions were performed following the method described previously (Virca).

#### cDNA cloning of human ABCA1

Based on sequence information of mouse ABCA1 cDNA we designed primers for RT-PCR analysis in order to amplify the human ABCA1 (ABCI) cDNA. Approximately 1  $\mu$ g of RNA from five day differentiated mononuclear phagocytes was reverse transcribed in a 20  $\mu$ l reaction using the RNA PCR Core Kit from Perkin Elmer. An aliquot of the cDNA was used in a 100  $\mu$ l PCR reaction performed with Amplitaq Gold (Perkin Elmer) and the following primer combinations: (primer names indicate the position in the corresponding mouse cDNA sequence):

*mABCI-144f* (5'-CAAACATGTCAGCTGTTACTGGA-3') and

*mABCI-643r* (5'-TAGCCTTGCAAA-AATACCTTCTG-3'),

*mABCI-1221f* (5'-GTTGGAAAGATTCTCTATACACCTG-3') and

*mABCI-1910r* (5'-CGTCAGCACTCTGATGATGGCCTG-3'),

*mABCI-3622f* (5'-TCTCTGCTATCTCCAACCTCA-3') and

*mABCI-4620r* (5'-ACGTCTTCACCAGGTAATCTGAA-3'),

*mABCI-5056f* (5'-CTATCTGTGTCATCTTTGCGATG-3') and

*mABCI-5857r* (5'-CGCTTCCTCCTATAGATCTTGGT-3'),

*mABCI-6093f* (5'-AAGAGAGCATGTGGA-GTTCTTTG-3') and

*mABCI-7051r* (5'-CCCTGTAATGGAATTGTGTTCTC-3'),

*hABCI-540f* (5'-AACCTTCTCTGGGTTCCTGTATC-3') and

*hABCI-1300r* (5'-AGTTCCTGGAA-GGTCTTGTTTAC-3'),

*hABCI-1831f* (5'-GCTGACCCCTTTGAGGACATGCG-3') and

5 *hABC1-3701r* (5'-ATAGGTCAGCTCATGCCCTATGT-3'),  
10 *hABC1-4532f* (5'-GCTGCC-TCCTCCACAAAGAAAAC-3') and  
*hABC1-5134r* (5'-GCTTTGCTGACCCGCTCC-TGGATC-3'),  
15 *hABC1-5800f* (5'-GAGGCCAGAATGACATCTTAGAA-3') and  
5 *hABC1-6259r* (5'-CTTGACAACACTTAGGGCACAAT-3').

20 All PCR products were cloned into the pUC18 plasmid vector and the nucleotide  
sequences were determined on a Pharmacia ALFexpress sequencer using the dideoxy  
chain-termination method and fluorescent dye-labeled primers.

10 Example 7

25 *Identification of the 5'end of ABCG1*

30 We could partially prove the 5'-end of ABCG1 published by Chen [7] that differs  
from the 5'-end published by Croop [6] obtained from the mRNA of human  
15 monocytes/macrophages using a 5' RACE approach. In detail the sequence according  
to Chen et al. downstream of position 25 was in agreement with our own data. In  
35 contrast, our identified sequence differs from the one reported by Chen [7] and Croop  
[6] at a site upstream of position 25 (Chen [7]). The sequence SEQ ID NO: 32 shows  
the newly identified 5'-end followed by the sequence published by Chen [7] from  
20 position 25.

45 *Molecular cloning and characterisation of the ABCG1 5'UTR*

50 We identified several fragments by screening of a  $\lambda$  phage library which contained a  
25 total of app. 3 kb of the 5' UTR upstream sequence of the human ABCG1 gene. The

sequence that comprises the 5'UTR and part of exon 1 (described above) are given in SEQ ID NO: 54.

The promoter activity of this sequence was proven by luciferase reporter gene assays in transiently transfected CHO cells.

Putative transcription factor binding sites within the promoter region with the highest likelihood ratio for the matched sequence as deduced from the TransFac database, GFB, Braunschweig, Germany. Multiple binding sites for SP-1, AP-1, AP-2 and CCAAT-binding factor (C/EBP family) are present within the first 1 kb of the putative promoter region.

Additionally, a transcription factor binding site involved in the regulation of apolipoprotein B was identified.

#### **Example 8**

##### **Characterization of the human ABCA8 full length cDNA**

The putative ABCA8 coding sequence is app. 6.5 kb in size. We successfully cloned and sequenced a 1kb segment of the human ABCA8 cDNA that encodes the putative second nucleotide binding site of the mature polypeptide (the sequence is shown in the sequence listing). The nucleotide sequence exhibits a 73% homology with the known human ABCA1 (ABC1) cDNA sequence.

We identified an alternative transcript in the cloned 1 kb coding region which consists of a 72 bp segment (see sequence listing). Genomic analysis of this region revealed that the alternative sequence is identical with a complete intron suggesting that the alternative mRNA is generated by intron retention. The retained intron introduces a preterminal stop codon and thus may code for a truncated ABCA8 variant.

ABCA8 also shows a cholesterol sensitive regulation of the mRNA expression (Table 2).

Tissue expression of ABCA8 is shown in table 1.

#### Example 9

##### Characterisation of the regulation of ABC transporter during differentiation of keratinocytic cells (HaCaT)

Differentiation of epidermal keratinocytes is accompanied by the synthesis of specific lipids composed mainly of sphingolipids (SL), free fatty acids (FFA), cholesterol (CH), and cholesterol sulfate, all involved in the establishment of the epidermal permeability barrier. The skin and, in particular, the proliferating layer of the epidermis is one of the most active sites of lipid synthesis in the entire organism. Cholesterol synthesis in normal human epidermis is LDL-independent, and circulating cholesterol levels do not affect the cutaneous de novo cholesterol synthesis. Fully differentiated normal human keratinocytes lack LDL receptors or its expression is very low, whereas in the normal human epidermis only basal cells express LDL receptors.

During keratinocyte differentiation a shift from polar glycerophospholipids to neutral lipids (FFA, TG) and also a replacement of short chain FFA by long chain highly saturated FFA is observed. The most important lipids for the barrier function of the skin are sphingolipids that account for one third of the lipids in the cornified layer, and consist of a large ceramide fraction as a result of glucosylceramide degradation by intercellular glycosidases and de novo synthesis of ceramide.

Glucosylceramide is synthesized intracellularly and stored in lamellar bodies and glucosylceramide synthase expression was found up-regulated during the differentiation of human keratinocytes.

Cholesterol sulfate is formed by the action of cholesterol sulfotransferase during keratinocyte differentiation. Cholesterol sulfate and the degrading enzyme steroid sulfatase are present in all viable epidermal layers, with the highest levels in the stratum granulosum. The gradient of cholesterol sulfate content across the stratum corneum (from inner to outer layers), and progressive desulfation of cholesterol sulfate regulate cell cohesiveness and normal stratum corneum keratinization and desquamation, respectively. Cholesterol sulfate induces transglutaminase 1 and the coordinate regulation of both factors is essential for normal keratinization.

The final step in lipid barrier formation involves lamellar body secretion and the subsequent post-secretory processing of polar lipids into their nonpolar lipid products through the action of hydrolytic enzymes that are simultaneously released ( $\beta$ -glucocerebrosidase, phospholipases, steroid sulfatase, acid sphingomyelinase). Disruption of the permeability barrier results in an increased cholesterol, fatty acid, and ceramide synthesis in the underlying epidermis. It has been shown that mRNA levels for the key enzymes required for cholesterol, fatty acid, and ceramide synthesis increased rapidly after artificial barrier disruption.

Currently the lipid transport systems in keratinocytes are poorly characterized. Several fatty acid transport related proteins have been identified in keratinocytes: plasma membrane fatty acid transport proteins (FATP) and intracellular fatty acid binding proteins (FABPs), most of them exhibiting high affinity for essential fatty acids. The expression of epidermal FABPs is up-regulated in hyperproliferative and inflammatory skin diseases, during keratinocyte differentiation and barrier disruption.

Based on our data on macrophages, we propose several ABC transporters as putative candidates for cellular lipid export in keratinocytes. We have examined the expression of all known ABC transporters during HaCaT cells differentiation. The human HaCaT cell line has a full epidermal differentiation capacity. Keratinocytes grown in

5 vitro as a monolayer at low calcium concentration ( $< 0.1 \text{ mM}$ ) can be differentiated  
by increasing calcium concentration in the culture medium ( $1-2 \text{ mM}$ ). We cultured  
10 HaCaT cells as a monolayer in calcium-free RMPI (Gibco) medium mixed with  
standard Ham's F12 medium at a ratio 3:1 supplemented with 10% chelex-treated  
5 FCS, Penicillin and Streptomycin. The final concentration of calcium in above  
medium was  $0.06 \text{ mM}$ . When the cells reached confluence (usually on 5<sup>th</sup> day of the  
15 culture), calcium concentration was enhanced up to the level of  $1.2 \text{ mM}$ . The cells  
were seeded at a density of  $2 \times 10^5 / \text{cm}^2$  in 60 mm culture dishes. The culture medium  
was replaced every two day and the cells were harvested after 24 h, 48 h, 4 d, 6 d,  
20 8 d and 10 d in culture, respectively. Total RNA from HaCaT cells was isolated  
using the isothiocyanate/cesium chloride-ultracentrifugation method.

25 The expression of all known human ABC transporters was examined during HaCaT  
cell differentiation (24 h, 48 h, 4 d, 6 d, 8 d, 10d, respectively) using a semi-  
15 quantitative RT-PCR approach (Table 6). The primer sets were generated from the  
published sequences of the ABC-transporters. Primers specific for GAPDH were  
used as a control. As a marker of keratinocyte differentiation CGT (ceramide  
30 glucosyl transferase) gene expression was assessed. Three of the transporters ex-  
amined, ABCB1 (MDR1), ABCB4 (MDR3), ABCD3 (PMP70), were not expressed.  
20 ABCC6 (MRP6), ABCA1 (ABC1), ABCD2 (ALDR and ABCB9 (est122234) were  
expressed at low levels (Table 6)

40 Most of the other transporters exhibited a biphasic expression pattern or were  
downregulated during keratinocyte differentiation. There was, however, a high  
25 expression of ABCG1 (ABC8), ABCA8 (new) and ABCC3 (MRP3) indicative for  
their involvement in terminal keratinocyte lipid secretion for cholesterol, FFAs and  
45 ceramide-backbone lipids. The two peroxisomal ABC transporters, ABCD2 (ALDR)  
and ABCD1 (ALDP) that mediate the transport of very long chain fatty acids into  
peroxisomes were initially expressed at relatively low levels and subsequently  
50 downregulated during differentiation. This is in agreement with the replacement of



5 short chain fatty acids by very long chain fatty acids during keratinocyte differentiation.

10 Example 10:

5 Sequencing of ABCA1 cDNA and genomic structure in five families of patients  
15 with Tangier disease revealed different mutations in the ABCA1 gene locus. These  
patients have different mutations at different positions in the ABCA1 gene, that  
result in changes in the protein structure of ABCA1. Family members that are  
20 heterozygous for these mutations show lowered levels of serum HDL, whereas the  
10 homocygote patients have extremely reduced HDL serum levels.

## Claims

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Claims:

1. A polynucleotide comprising a member selected from the group consisting of:
  - (a) a polynucleotide encoding the polypeptide as set forth in SEQ ID NO:2;
  - (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and
  - (c) a polynucleotide fragment of the polynucleotide of (a) or (b).
2. The polynucleotide of claim 1 wherein the polynucleotide is DNA.
3. A vector containing one or more of the polynucleotides of claim 1 and 2.
4. A host cell containing the vector of claim 3.
5. A process for producing a polypeptide comprising: expressing from the host cell of claim 4 the polypeptide encoded by said DNA.
6. A polypeptide selected from the group consisting of
  - (a) a polypeptide having the deduced amino acid sequence of SEQ ID NO:2 and fragments, analogs and derivatives thereof, and
  - (b) a polypeptide comprising amino acid 1 to amino acid 2201 of SEQ ID NO:2.
7. An antibody capable to bind to the polypeptide of claim 6.
8. A diagnostic kit for the detection of the polypeptide of claim 6.

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9. Use of a polypeptides encoded by a polynucleotide comprising a member selected from the group consisting of:

- 5
- (a) a polynucleotide as set forth in SEQ ID NO:1, 3, 4 and 6 to 31;
  - (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and
  - (c) a polynucleotide fragment of the polynucleotide of (a) or (b)

in an assay for for detecting modulators of said polypeptides.

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10. Modulator of a polypeptides encoded by a polynucleotide comprising a member selected from the group consisting of:

- 15
- (a) a polynucleotide as set forth in SEQ ID NO:1, 3, 4 and 6 to 31;
  - (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and
  - (d) a polynucleotide fragment of the polynucleotide of (a) or (b)

11. A pharmaceutical comprising the modulator of claim 10

12. An assay for detecting polypeptides encoded by a polynucleotide comprising a member selected from the group consisting of:

- 25
- (a) a polynucleotide as set forth in SEQ ID NO:1, 3, 4 and 6 to 32 and 54;
  - (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and
  - (c) a polynucleotide fragment of the polynucleotide of (a) or (b)

Figure 1

2588 GA TCAATCGCAT TCATTTTAAG AAATTATACC TTTTAGTAC TTGCTGAAGA  
2641 ATGATTCAGG GTAAATCACA TACTTTGTTT AGAGAGGCGA GGGGTTTAAC CCGAGTCACC  
2701 CAGCTGGTCT CATACATAGA CAGCACTTGT GAAGGATTGA ATGCAGGTTT CAGGTGGAGG  
2761 GAAGACGTGG ACACCATCTC CACTGAGCCA TGCAGACATT TTTAAAAGCT ATACACAAAA  
2821 TTGTGAGAAG ACATTGGCCA ACTCTTTCA AGTCTTTCTT TTTCCACGTG CTTCTTATTT  
2881 TAAGCGAAT ATATTGTTG TTTCTCCTA AAAAAAAAAA 2890

Figure 2

1 CAAACATGTCAGCTGTTACTGGAAGTGGCCTGGCCTCTATTTATCTTCCTGATCCTGATC 60  
61 TCTGTTCGGCTGAGCTACCCACCCTATGAACAACATGAATGCCATTTTCCAAATAAAGCC 120  
121 ATGCCCTCTGCAGGAACACTTCCTTGGGTTTCAGGGATTATCTGTAATGCCAACACCCCC 180  
1 M P S A G T L P W V Q G I I C N A N N P 20  
181 TGTTCCGTTACCCGACTCCTGGGGAGGCTCCCGGAGTTGTTGGAACTTTAACAATCC 240  
21 C F R Y P T P G E A P G V V G N F N K S 40  
241 ATTGTGGCTCGCCTGTTCTCAGATGCTCGGAGGCTTCTTTTATACAGCCAGAAAGACACC 300  
41 I V A R L F S D A R R L L L Y S Q K D T 60  
301 AGCATGAAGGACATGCCCAAAGTTCTGAGAACATTACAGCAGATCAAGAAATCCAGCTCA 360  
61 S M K D M R K V L R T L Q Q I K K S S S 80  
361 AACTTGAAGCTTCAAGATTTCCTGGTGGACAATGAAACCTTCTCTGGGTTCTCTGTATCAC 420  
81 N L K L Q D F L V D N E T F S G F L Y H 100  
421 AACCTCTCTCTCCCAAAGTCTACTGTGGACAAGATGCTGAGGGCTGATGTCATTCTCCAC 480  
101 N L S L P K S T V D K M L R A D V I L H 120  
481 AAGGTATTTTGGCAAGGCTACCACTTACATTTGACAAGTCTGTGCAATGGATCAAAATCA 540  
121 K V F L Q G Y Q L H L T S L C N G S K S 140  
541 GAAGAGATGATTCAACTTGGTGACCAAGAAGTTTCTGAGCTTTGTGGCCTACCAAGGGAG 600  
141 E E M I Q L G D Q E V S E L C G L P R E 160  
601 AAAGTGGCTGCAGCAGAGCGAGTACTTCGTTCCAACATGGACATCCTGAAGCCAATCCTG 660  
161 K L A A A E R V L R S N M D I L K P I L 180  
661 AGAAGCTAAACTCTACATCTCCCTTCCCGAGCAAGGAGCTGGCCGAAGCCACAAAAACA 720  
181 R T L N S T S P F P S K E L A E A T K T 200  
721 TTGCTGCATAGTCTTGGGACTCTGGCCAGGAGCTGTTTCAGCATGAGAAGCTGGAGTGAC 780  
201 L L H S L G T L A Q E L F S M R S W S D 220  
781 ATGCGACAGGAGGTGATGTTTCTGACCAATGTGAACAGCTCCAGCTCCTCCACCCAAATC 840  
221 M R Q E V M F L T N V N S S S S S S T Q I 240  
841 TACCAGGCTGTCTCTGTTCTGCTGGGGCATCCCGAGGGAGGGGGGCTGAAGATCAAG 900  
241 Y Q A V S R I V C G H P E G G G L K I K 260  
901 TCTCTCAACTGGTATGAGGACAACAACCTACAAGCCCTCTTTGGAGGCAATGGCACTGAG 960  
261 S L N W Y E D N N Y K A L F G G N G T E 280

961 GAAGATGCTGAAACCTTCTATGACAACTCTACAACCTCCTTACTGCAATGATTTGATGAAG 1020  
281 E D A E T F Y D N S T T P Y C N D L M K 300  
1021 AATTTGAGTCTAGTCCTCTTTCCCGCATTATCTGGAAAGCTCTGAAGCCGCTGCTCGTT 1080  
301 N L E S S P L S R I I W K A L K P L L V 320  
1081 GGAAGATCCTGTATACACCTGACACTCCAGCCACAAGGCAGGTCATGGCTGAGGTGAAC 1140  
321 G K I L Y T P D T P A T R Q V M A E V N 340  
1141 AAGACCTTCCAGGAAGCTGGCTGTGTTCATGATCTGGAAGGCATGTGGGAGGAAGTCAAGC 1200  
341 K T F Q E L A V F H D L E G M W E E L S 360  
1201 CCCAAGATCTGGACCTTTCATGGAGAAGCAAGAAATGGACCTTGTCCGGATGCTGTTG 1260  
361 P K I W T F M E N S Q E M D L V R M L L 380  
1261 GACAGCAGGGACAATGACCACTTTTGGGAACAGCAGTTGGATGGCTTAGATTGGACAGCC 1320  
381 D S R D N D H F W E Q Q L D G L D W T A 400  
1321 CAAGACATCGTGGCGTTTTTGGCCAAGCAGCCAGAGGATGTCCAGTCCAGTAATGCTTCT 1380  
401 Q D I V A F L A K H P E D V Q S S N G S 420  
1381 GTGTACACCTGGAGAGAAGCTTTCAACGAGACTAACCAGGCAATCCGGACCATATCTCGC 1440  
421 V Y T W R E A F N E T N Q A I R T I S R 440  
1441 TTCATGGAGTGTGTCAACCTGAACAAGCTAGAACCATAGCAACAGAAGTCTGGCTCATC 1500  
441 F M E C V N L N K L E P I A T E V W L I 460  
1501 AACAAAGTCCATGGAGCTGTGGATGAGAGGAAGTTCTGGGCTGGTATTGTGTTCACTGGA 1560  
461 N K S M E L L D E R K F W A G I V F T G 480  
1561 ATTACTCCAGGCAGCATTGAGCTGCCCCATCATGTCAAGTACAAGATCCGAATGGACATT 1620  
481 I T P G S I E L P H H V K Y K I R M D I 500  
1621 GACAATGTGGAGAGGACAAATAAATCAAGGATGGGTACTGGGACCCTGGTCTCGAGCT 1680  
501 D N V E R T N K I K D G Y W D P G P R A 520  
1681 GACCCCTTTGAGGACATGCGGTACGTCTGGGGGGGCTTCGCCTACTTGCAGGATGTGGTG 1740  
521 D P F E D M R Y V W G G F A Y L Q D V V 540  
1741 GAGCAGGCAATCATCAGGCTGCTGACGGGCACCGAGAAGAAAAGTGGTGTCTATATGCAA 1800  
541 E Q A I I R V L T G T E K K T G V Y M Q 560  
1801 CAGATGCCCTATCCCTGTTACGTTGATGACATCTTCTCGGGGTGATGAGCCGGTCAATG 1860  
561 Q M P Y P C Y V D D I F L R V M S R S M 580  
1861 CCCCTCTTCATGACGCTGGCCTGGATTACTCAGTGGCTGTGATCATCAAGGGCATCGTG 1920  
581 P L F M T L A W I Y S V A V I I K G I V 600  
1921 TATGAGAAGGAGGCACGGCTGAAAGAGACCATGCGGATCATGGCCTGGACAACAGCATC 1980  
601 Y E K E A R L K E T M R I M G L D N S I 620  
1981 CTCTGGTTTAGCTGGTTTATTAGTACCTCATTCTCTTCTTGTGAGCGCTGGCCTGCTA 2040  
621 L W F S W F I S S L I P L L V S A G L L 640  
2041 GTGGTCACTCCTGAAGTTAGGAAACCTGCTGCCCTACAGTGATCCAGCGTGGTGTGTC 2100  
641 V V I L K L G N L L P Y S D P S V V F V 660  
  
2101 TTCCTGTCCGTGTTTGCTGTGCTGACAATCCTGCAGTGCTTCCTGATTAGCACACTCTTC 2160

661 F L S V F A V V T I L Q C F L I S T L F 680  
2161 TCCAGAGCCAACCTGGCAGCAGCCTGTGGGGGCATCATCTACTTCACGCTGTACCTGCCC 2220  
681 S R A N L A A A C G G I I Y F T L Y L P 700  
2221 TACGTCCTGTGTGTGGCATGGCAGGACTACGTGGGCTTCACACTCAAGATCTTCGCTAGC 2280  
701 Y V L C V A W Q D Y V G F T L K I F A S 720  
2281 CTGCTGTCTCCTGTGGCTTTTGGGTTTGGCTGTGAGTACTTTGCCCTTTTGGAGGAGCAG 2340  
721 L L S P V A F G F G C E Y F A L F E E Q 740  
2341 GGCATTGGAGTGCAGTGGGACAACCTGTTGAGAGTCTGTGGAGGAAGATGGCTTCAAT 2400  
741 G I G V Q W D N L F E S P V E E D G F N 760  
2401 CTCACCACTTCGGTCTCCATGATGCTGTTTGACACCTTCCTCTATGGGGTGATGACCTGG 2460  
761 L T T S V S M M L F D T F L Y G V M T W 780  
2461 TACATTGAGGCTGTCTTTCCAGGCCAGTACGGAATCCCGAGCCCTGGTATTTTCCTTGC 2520  
781 Y I E A V F P G Q Y G I P R P W Y F P C 800  
2521 ACCAAGTCCTACTGTTTGGCGAGGAAAGTGATGAGAAGAGCCACCCTGGTTCACACAG 2580  
801 T K S Y W F G E E S D E K S H P G S N Q 820  
2581 AAGAGAATATCAGAAATCTGCATGGAGGAGGAACCCACCCACTGAAGCTGGGCGTGTCC 2640  
821 K R I S E I C M E E E P T H L K L G V S 840  
2641 ATTCAGAACCTGGTAAAGTCTACCGAGATGGGATGAAGGTGGCTGTGATGGCCTGGCA 2700  
841 I Q N L V K V Y R D G M K V A V D G L A 860  
2701 CTGAATTTTATGAGGGCCAGATCACCTCCTTCCTGGGCCACAATGGAGCGGGGAAGACG 2760  
861 L N F Y E C Q I T S F L G H N G A G K T 880  
2761 ACCACCATGTCAATCCTGACCGGGTGTTCCTCCCCGACCTCGGGCACCGCCTACATCCTG 2820  
881 T T M S I L T G L F P P T S G T A Y I L 900  
2821 GGAAAGACATTGCTCTGAGATGAGCACCATCCGGCAGAACCTGGGGGTCTGTCCCCCAG 2880  
901 G K D I R S E M S T I R Q N L G V C P Q 920  
2881 CATAACGTGCTGTTTACATGCTGACTGTGGAAGAACACATCTGGTCTATGCCCCGCTTG 2940  
921 H N V L F D M L T V E E H I W F Y A R L 940  
2941 AAAGGGCTCTCTGAGAAGCACGTGAAGGCGGAGATGGAGCAGATGGCCCTGCATGTTGGT 3000  
941 K G L S E K H V K A E M E Q M A L D V G 960  
3001 TTGCCATCAAGCAACCTGAAAAGCAAAACAAGCCAGCTGTCAGGTGGAATGCAGAGAAAG 3060  
961 L P S S K L K S K T S Q L S G G M Q R K 980  
3061 CTATCTGTGGCCTTGGCCTTTGTGGGGGATCTAAGGTTGTCTTCTGGATGAACCCACA 3120  
981 L S V A L A F V G G S K V V I L D E P T 1000  
3121 GCTGGTGTGGACCCTTACTCCCGCAGGGGAATATGGGAGCTGCTGCTGAAATACCGACAA 3180  
1001 A G V D P Y S R R G I W E L L L K Y R Q 1020  
3181 GGCCGCACCATTTATCTCTCTACACACCACATGGATGAAGCGGACGCTCCTGGGGACAGG 3240  
1021 G R T I I L S T H H M D E A D V L G D R 1040  
3241 ATTGCCATCATCTCCCATGGGAAGCTGTGCTGTGTGGGCTCCTCCCTGTTTCTGAAGAAC 3300  
1041 I A I I S H G K L C C V G S S L F L K N 1060  
3301 CAGCTGGGAACAGGCTACTACCTGACCTTGGTCAAGAAAGATCTGGAATCCTCCCTCAGT 3360

1061 Q L G T G Y Y L T L V K K D V E S S L S 1080  
3361 TCCTGCAGAAACAGTAGTAGCACTGTGTCATACCTGAAAAAGGAGGACAGTGTTCCTCAG 3420  
1081 S C R N S S S T V S Y L K K E D S V S Q 1100  
3421 AGCAGTTCTGATGCTGGCCTGGGCAGCGACCATGAGAGTGACACGCTGACCATCGATGTC 3480  
1101 S S S D A G L G S D H E S D T L T I D V 1120  
3481 TCTGCTATCTCCAACCTCATCAGGAAGCATGTGTCTGAAGCCCGGCTGGTGAAGACATA 3540  
1121 S A I S N L I R K H V S E A R L V E D I 1140  
3541 GGGCATGAGCTGACCTATGTGCTGCCATATGAAGCTGCTAAGGAGGGAGCCTTTGTGGAA 3600  
1141 G H E L T Y V L P Y E A A K E G A F V E 1160  
3601 CTCTTTTCATGAGATTGATGACCGGCTCTCAGACCTGGGCATTCTAGTTATGGCATCTCA 3660  
1161 L F H E I D D R L S D L G I S S Y G I S 1180  
3661 GAGACGACCTGGAAGAAATATTCCTCAAGGTGGCCGAAGAGAGTGGGGTGGATGCTGAG 3720  
1181 E T T L E E I F L K V A E E S G V D A E 1200  
3721 ACCTCAGATGGTACCTTGCCAGCAAGACGAAACAGGCGGGCCTTCGGGGACAAGCAGAGC 3780  
1201 T S D G T L P A R R N R R A F G D K Q S 1220  
3781 TGTCTTCGCCCGTTCACTGAAGATGATGCTGCTGATCCAAATGATTCTGACATAGACCCA 3840  
1221 C L R P F T E D D A A D P N D S D I D P 1240  
3841 GAATCCAGAGAGACAGACTTGCTCAGTGGGATGGATGGCAAAGGCTCTACCAGGTGAAA 3900  
1241 E S R E T D L L S G M D G K G S Y Q V K 1260  
3901 GGCTGGAACCTTACACAGCAACAGATTGTGGCCCTTTTGTGGAAGAGACTGCTAATGCC 3960  
1261 G W K L T Q Q Q F V A L L W K R L L I A 1280  
3961 AGACGGAGTCGAAAGGATTTTTGCTCAGATTGTCTTGCCAGCTGTGTTTGTCTGCATT 4020  
1281 R R S R K G F F A Q I V L P A V F V C I 1300  
4021 GGCCTGTGTTCAGCCTGATCGTGCCACCCCTTTGGCAAGTACCCAGCCTGGAACCTCAG 4080  
1301 A L V F S L I V P P F G K Y P S L E L Q 1320  
4081 CCCTGGATGTACAACGAACAGTACACATTTGTGCAATGATGCTCCTGAGGACACGGGA 4140  
1321 P W M Y N E Q Y T F V S N D A P E D T G 1340  
4141 ACCCTGGAACCTCTTAAACGCCCTCACCAAGACCCCTGGCTTCGGGACCCGCTGTATGGAA 4200  
1341 T L E L L N A L T K D P G F G T R C M E 1360  
4201 GGAAACCCAAATCCAGACACGCCCTGCCAGGCAGGGGAGGAAGAGTGGACCACTGCCCCA 4260  
1361 G N P I P D T P C Q A G E E E W T T A P 1380  
4261 GTTCCCCAGACCATCATGGACCTCTTCCAGAATGGGAACCTGGACAATGCAGAACCCTTCA 4320  
1381 V P Q T I M D L F Q N G N W T M Q N P S 1400  
4321 CCTGCATGCCAGTGTAGCAGCGACAAAATCAAGAAGATGCTGCCTGTGTGCCCCCAGGG 4380  
1401 P A C Q C S S D K I K K M L P V C P P G 1420  
4381 GCAGGGGGGCTGCCCTCCTCCACAAAGAAAAACAAACACTGCAGATATCCTTCAGGACCTG 4440  
1421 A G G L P P P Q R K Q N T A D I L Q D L 1440  
4441 ACAGGAAGAAACATTTCCGATTATCTGGTGAAGACGTATGTGCAGATCATAGCCAAAAGC 4500  
1441 T G R N I S D Y L V K T Y V Q I I A K S 1460  
4501 TTAAAGAACAAGATCTGGGTGAATGAGTTAGGTATGGCGGCTTTCCCTGGGTGTCACT 4560



1461 L K N K I W V N E F R Y G G F S L G V S 1480  
4561 AATACTCAAGCACTTCCTCCGAGTCAAGAAGTTAATGATGCCACCAAACAAATGAAGAAA 4620  
1481 N T Q A L P P S Q E V N D A T K Q M K K 1500  
4621 CACCTAAAGCTGGCCAAGCACAGTTCTGCAGATCGATTTCTCAACAGCTTGGGAAGATTT 4680  
1501 H L K L A K D S S A D R F L N S L G R F 1520  
4681 ATGACAGGACTGGACACCAGAAATAATGTCAAGGTGTGGTTCAATAACAAGGGCTGGCAT 4770  
1521 M T G L D T R N N V K V W F N N K G W H 1540  
4741 GCAATCAGCTCTTCTCCTGAATGTCAACAATGCCATTCTCCGGGCCAACCTGCAAAAAG 4800  
1541 A I S S F L N V I N N A I L R A N L Q K 1560  
4801 GGAGAGAACCCTAGCCATTATGGAATTACTGCTTCAATCATCCCTGAATCTCACCAAG 4860  
1561 G E N P S H Y G I T A F N H P L N L T K 1580  
4861 CAGCAGCTCTCAGAGGTGGCTCCGATGACCACATCAGTGGATGTCTTGTGTCCATCTGT 4920  
1581 Q Q L S E V A P M T T S V D V L V S I C 1600  
4921 GTCATCTTTGCAATGTCTCCTCCGAGCCAGCTTTGTCTGATTCTCTGATCCAGGAGCGG 4980  
1601 V I F A M S F V P A S F V V F L I Q E R 1620  
4981 GTCAGCAAAGCAAACACCTGCAGTTCATCAGTGGAGTGAAGCCTGTCTCTACTGGCTC 5040  
1621 V S K A K H L Q F I S G V K P V I Y W L 1640  
5041 TCTAATTTTGTCTGGGATATGTGCAATTACGTTGTCCCTGCCACACTGGTCATTATCATC 5100  
1641 S N F V W D M C N Y V V P A T L V I I I 1660  
5101 TTCATCTGCTTCCAGCAGAAGTCTATGTGTCTCCACCAATCTGCCTGTGCTAGCCCTT 5160  
1661 F I C F Q Q K S Y V S S T N L P V L A L 1680  
5161 CTACTTTTGTCTATGGGTGGTCAATCACACCTCTCATGTACCCAGCCTCCTTTGTGTTT 5220  
1681 L L L L Y G W S I T P L M Y P A S F V F 1700  
5221 AAGATCCCCAGCACAGCCTATGTGGTGGTCAATCAGCCTGAACCTCTTCATTGGCATTAA 5280  
1701 K I P S T A Y V V L T S V N L F I G I N 1720  
5281 GGCAGCGTGGCCACCTTTGTGCTGGAGCTGTTCCCGACAATAAGCTGAATAATATCAAT 5340  
1721 G S V A T F V L E L F T D N K L N N I N 1740  
5341 GATATCCTGAAGTCCGTGTTCTTGATCTCCACATTTTGCCTGGGACGAGGGCTCATC 5400  
1741 D I L K S V F L I F P H F C L G R G L I 1760  
5401 GACATGGTGA AAAAACCAGGCAATGGCTGATGCCCTGGAAAGGTTTGGGGAGAATCGCTTT 5460  
1761 D M V K N Q A M A D A L E R F G E N R F 1780  
5461 GTGTCAACATTATCTTGGGACTTGGTGGGACGAAACCTCTTCGCCATGGCCGTGGAAGGG 5520  
1781 V S P L S W D L V G R N L F A M A V E G 1800  
5521 GTGGTGTCTTCTCCTCATTACTGTCTGATCCAGTACAGATTCTTCATCAGGCCCCAGACCT 5580  
1801 V V F F L I T V L I Q Y R F F I R P R P 1820  
5581 GTAAATGCAAAGCTATCTCCTCTGAATGATGAAGATGAAGATGTGAGGCGGGAAGACAG 5640  
1821 V N A K L S P L N D E D E D V R R E R Q 1840  
5641 AGAATTCTTGATGGTGGAGGCCAGAATGACATCTTAGAAATCAAGGAGTTGACGAAGATA 5700  
1841 R I L D G G G Q N D I L E I K E L T K I 1860  
5701 TATAGAAGGAAGCGGAAGCCTGCTGTTGACAGGATTTCCGTGGGCATTCTCTCTGGTGAG 5760

1861 Y R R K R K P A V D R I C V G I P P G E 1880  
 5761 TGCTTTGGGCTCCTGGGAGTTAATGGGGCTGGAAAATCATCAACTTTCAAGATGTTAACA 5820  
 1881 C F G L L G V N G A G K S S T F K M L T 1900  
 5821 GGAGATACCACTGTTACCAGAGGAGATGCTTTCCTTAACAGAAATAGTATCTTATCAAAC 5880  
 1901 G D T T V T R G D A F L N R N S I L S N 1920  
 5881 ATCCATGAAGTACATCAGAACATGGGCTACTGCCCTCAGTTTGATGCCATCAGAGCTG 5940  
 1921 I H E V H Q N M G Y C P Q F D A I T E L 1940  
 5941 TTGACTGGGAGAGAACACGTGGAGTTCTTTGCCCTTTTGAGAGGAGTCCCAGAGAAAGAA 6000  
 1941 L T G R E H V E F F A L L R G V P E K E 1960  
 6001 GTTGGCAAGCTTGGTGAGTGGGCGATTTCGGAACCTGGGCCCTCGTGAAGTATGGAGAAAA 6060  
 1961 V G K V G E W A I R K L G L V K Y G E K 1980  
 6061 TATGCTGGTAACATAGTGGAGGCAACAAACGCAAGCTCTCTACAGCCATGGCTTTGATC 6120  
 1981 Y A G N Y S G G N K R K L S T A M A L I 2000  
 6121 GCGGGGCTCCTGTGGTGTCTTCTGCAATGAACCCACACAGGCATGGATCCCAAAGCCCGG 6180  
 2001 G G P P V V F L D E P T T G M D P K A R 2020  
 6181 CGGTCTTGTGGAATTGTGCCCTAAGTGTGTCTCAAGGAGGGGAGATCAGTAGTGCTTACA 6240  
 2021 R F L W N C A L S V V K E G R S V V L T 2040  
 6241 TCTCATAGTATGGAAGAATGTGAAGCTCTTTGCACTAGGATGGCAATCATGGTCAATGGA 6300  
 2041 S H S M E E C E A L C T R M A I M V N G 2060  
 6301 AGGTTCAAGTGCCCTGGCAGTGTCCAGCATCTAAAAAATAGGTTTGGAGATGGTTATACA 6360  
 2061 R F R C L G S V Q H L K N R F G D G Y T 2080  
 6361 ATAGTTGTACGAATAGCAGGGTCCAACCCGACCTCAAGCCTGTCCAGGATTCTTTTGA 6420  
 2081 I V V R I A G S N P D L K P V Q D F F G 2100  
 6421 CTTGCATTTCTGGAAGTGTTCAAAAGAGAAAACACCGGAACATGCTACAATACAGCTT 6480  
 2101 L A F P G S V P K E K H R N M L Q Y Q L 2120  
 6481 CCATCTTCATTATCTTCTCTGGCCAGGATATTTCAGCATCCTCTCCAGAGCAAAAAGCGA 6540  
 2121 P S S L S S L A R I F S I L S Q S K K R 2140  
 6541 CTCCACATAGAAGACTACTCTGTTTCTCAGACAACACTTGACCAAGTATTTGTGAACCTT 6600  
 2141 L H I E D Y S V S Q T T L D Q V F V N F 2160  
 6601 GCCAAGGACCAAAGTGATGATGACCACTTAAAAGACCTCTCATTACACAAAACCAGACA 6660  
 2161 A K D Q S D D D H L K D L S L H K N Q T 2180  
 6661 GTAGTGGACGTTGCAGTTCTCACATCTTTTCTACAGGATGAGAAAGTGAAAGAAAGCTAT 6720  
 2181 V V D V A V L T S F L Q D E K V K E S Y 2200  
 6721 GTATGAAGAATCCTGTTTCATACGGGGTGGCTGAAAGTAAAGAGGGACTAGACTTTCCTTT 6780  
 2201 V \*  
 6781 GCACCATGTGAAGTGTGTGGAGAAAAGAGCCAGAAGTTGATGTGGGAAGAAGTAAACTG 6840  
 6841 GATACTGTACTGATACTATTCAATGCAATGCAATTCAATG 6880

Figure 3

5' 1 GTACCCCCCT TGCTGGTTG ATCCTCAGGG TTCTACTTAG AATGCCTCGA

51 AAAGTCTTGG CTGGACACCC ATGCCCAGTC TTTCTGCAGG GTCCCATTGG  
101 GGTTAACCTT CTCATTTCAT CCCATGTGAA CCAGGCCAGG CCCATCAGGG  
151 TTTGGCAACC CCCTGATGCA GTGGTTGCTG CCAGGTGACA GGAGCAAGCC  
201 TGCAGCTGCT GGGGGGCCAT GCAGAGACAG CCTGCCAGAG GGGAGACCAC  
251 CTGGGGAGGC CAGAGCCGTG GAGACAGCAA GAGACCAGGG GCTGAGGACA  
301 GAGTAGTACA GGTCTTTGGT CCCAGTAGTC CTGAAACCAC TGCACTCCGA  
351 ACCTTTCTGT ACTTAGCTTA AGCCAGTTGG AGTTTCTGTC CTTTACAACC  
401 AAGAGCCTTG ATAGGAATGG GGTCTGTGTC TACGCTACTG TTGGCTTCTT  
451 TCCCGATCGG GCGCTGGAGG GGAACACAGC AGTGACTACA GTGGGATGCT  
501 TACTCGGTGC TGGGCATGCT AGAAAGTGCT TGCCATGCCT TATTTCCAC  
551 GTGGTGGGA TTTTGACCCC ACCTGTACAG ACAGATAAGT GAGGACCCTT  
601 TTCACCTTAT CCTGCAACAG AAAATCCAGC AGCCAAAGCC AACAAGGGCC  
651 CAGCATAGCA TCTTCCCTCT CTGACTTCAT CCTCACGCTC CACACACCAT  
701 CCCCCTGCC ATTCCCAGCA GCCCAGTAAG CACTGCCTCA CACTTCCAGT  
751 TCCGGACCAG CCAGGATGGC CAGGCTGGAT GGGGGCCATC CACCGGCTGA  
801 AGCCAATTGC CTATTCTCGA GCTGAAGGTG AATCAATCCC GCATAAATCT  
851 TCGGGCAGAG AACTNNGGTG GGGGGTAGAA GAGGGGAAT GTCTAGAAGG  
901 AAATTCTGGG GCACATTCCT GGAAGTGAGG AGGATGGATA TTGGACAGAA  
951 ATTATGTCAT TGCAGGCACC CTCACTTGCC CTGGCCACAT GGACAGTTCC  
1001 TCCCCGGCTG TGTTCGNGC CTCCTCTCGT GCTCCAGGGC CTGTCTGTTT  
1051 CTGGAGCGAG ATGGGTCCCA GGGCTGGGCA CCAGTCCCA TCTCCAGCCA  
1101 TCAGGCACCTT TCCTCTCTGT GTTTTGGCGT AAACACNTCC CTAGGTTTGT  
1151 GGATCTGAAT CCTCTTCCCA ACACACTCAA GCTTTGCTGG GCCTCCCTGC  
1201 AGTGTATGTT TAAGGCACCA CACAGCCTCC AAGGCCTGGC ACCCGGGCAG  
1251 TGGCCACCTG GTAAACACAG CAGTCAGATT TCCTCATTTC AGCCAAGTGT  
1301 AAAATCAAGG TAATGGATCT ACNCTTTTTT TTTTNTNTTT TTTCCAGGGG  
1351 GNTNNTTTTT TTTTGAGACG GAGTCTCACT CTGTCANCCC CGGTCTGGAG  
1401 TGCAGTGGCT CAATCTCGGC TCANCTGGCA AGCTCCGCCT CCCAGTTCA  
1451 TGCCATTCTC CTGCCTCAGC CTACATAGTA GCTGGGACTA CAGGTGCCCCG  
1501 CCACCACACC TAGCTAATTT TTTGTATTTT TAGTAGAGAC GGGGTTTCAT  
1551 CATGTTAGCC AGGATGGTCT CGATCTCCTG ACCTCCCAA GTGGTGGGAG  
1601 TTACAGGTGT GAGCCACTGC GCNCCGGCTG GATGACTCTT GAGACAACAC  
1651 CATTAGACA AAGGCAAGGC CTCCCCTTA AACTCATAAC CGTGTCTCCT  
1701 TTCTCTCCTT CGATTTGAGC GGCTGAATTT GGTTACAGTC ATCTGACCTG  
1751 TGGGTGTGAA NGTCCACCTG CCTGGCATAA AAAGCTGTGC CTCCTTTCTA  
1801 GGTGAGGAGA AAGAGAGAGA CCTGGTCAT CTGAGGTGTG GTTGGGAGGG  
1851 GGGACCCAGG TGTGCTGGAA ATGAAAAGAA ATGCATTCTT GTTTTTTCGT  
1901 CCAACATGC AAACAACCTGA ACAAAGCAT TAGGGCCTGA GACTGGGAGT  
1951 AAAGAATTCC TTGTCACCAT GGATACCAGG AAATGGCCCC ACTTATATAT  
2001 AATAAGGGCT TTAGAGATGC TGGACCATCT GATATTCCAG CCTGGGGCCA  
2051 CATGGGAGTG TGCCCTGGTG TTATTCCTTA TACAGTTCCA TGAACATGGC  
2101 TCTGAAACA CCTCTGTCTG CAGAAAATGA GGCTTTTCTT TTTTGTTCG

2151 GGGGTGAACA GAGGGCAGAG GCCTGGGCAT CTTCACTCAG CACCCCTTTG  
 2201 TAACCCAGCA CTTAGCACCA TGGCTGGCGC ACAGCAATGT CACATGTGTG  
 2251 AGTGCACACG ATGCCTCACT GCCAGGGGTC ACCCCACACC GGTGCTGTTG  
 2301 GGGGCGTTGG AGTGGTTATC TCTTCTTTAG TCCTCAAGCT CCTACCTGGC  
 2351 AGAGAGCTGC CCAACACCGT CGGGGTGGGG TGGGCGGGAA GGAAGAAGC  
 2401 AGCAGCAAGA AAGAAGCCCC CTGGCCCTCA CTCTCCCTCC CTGGACGCC  
 2451 CCTCTTCGAC CCCATCACAC AGCCGCTTGA GCCTTGGAGN CAGTGGATT  
 2501 CCGAGCCTGG GAACCCCGG CGTCTGTCCC GGTGTCCCC GCAGCCTCAC  
 2551 CCNCGTGTG GCCAGCCCC CGCGAGTTCG GGACCCGGGG TTTCCGGGGT  
 2601 GGCAGGGGGT TCCCATGCCG CTGCGAGGC CTCGGCTCGG GCCGCTCCCG  
 2651 GAACCTGCAC TTCAGGGGTC CTGGTCCGCC GCCCCAGCA GGAGCAAAAC  
 2701 AAGAGCACGC GCACCTGCCG GCCCGCCCGC CCCCTTGGTG CCGGCCAATC  
 2751 GCGCGCTCGG GCGGGGGTCG GCGCGCTGG AACAGAGCC GGAGCCGGAT  
 2801 CCCAGCCGGA GCCCAAGCGC AGCCCGCACC CCGCGCAGCG GCTGAGCCGG  
 2851 GAGCCAGCGC AGCCTCGGCC CCGCAGCTCA AGCCTCGTCC CCGCCGCCNG  
 2901 CCGCCGCACG CCGCCGCCGC CGCCCCGGG GCATGGCTGT CTGATGGCCG

## EXON1/INTRON 1

2951 CTTTCTCGGT CGGCACCGCC ATGGTGAGTG AGCGCATCCT TCGTCCGCCG  
 3001 GGAACGGTTT TATTTTCAAG GAGAGCAGGA AACACACAAA GACTCGCAAG  
 3051 CTCGACCTGA CACCCCTCCC AGGAGCGCGT CCTCTGGGGC GCTGACCCAG  
 3101 GGGCACCTTA GAGTGGCGCC CGGCTCCGAT CGCTGCCCTT NNCCCTCCG  
 3151 CCAGGGCCAC CTGGGAGCCT CGGGGATGCC CCTTGACCG GCAGAGNGCA  
 3201 CGGACTAGGT GGAGGGGNCC GGGATTGGGG CGGGGGGAGC NCAGTTGCCC  
 3251 TACAAGTTGG ACCGATGGCC TTGACCTGAT GGCTTCTGGG CCGGGGGCGT  
 3301 GGGGAGCTGG GGACCCGAG CGCACTGGGG ACTGGGGAGG GGCCGAGCT  
 3351 TGGGCCGAG GGAAGAGGGG ACTTGAAGAA GGGGAGCCCC GCGCGCGCGG  
 3401 CTGTGGGCTT GGGGACCGGG GACTTCTGCG GCCATCCCCA GGAACGCCAG  
 3451 GCAAGGTCTG GGAACAAAA GAGGAAGCTG CCCCAGAGA GCCGGAGCTC  
 3501 GACTGNACTC CC 3'

Figure 4

5'

1 CTTGGTGCCG CATGCATCGT GGTGCTCATC TTTCTGGCCT TCCAGCAGAG  
 51 GGCATATGTG GCCCCTGCCA ACCTGCCTGC TCTCCTGCTG TTGCTACTAC  
 101 TGTATGGCTG GTCGATCACA CCGCTCATGT ACCCAGCCTC CTTCTTCTTC  
 151 TCCGTGCCCA GCACAGCCTA TGTGGTGCTC ACCTGCATAA ACCTCTTTAT  
 201 TGGCATCAAT GGAAGCATGG CCACCTTTGT GCTTGAGCTC TTCTCTGATC  
 251 AGAAGCTGCA GGAGGTGAGC CGGATCTTGA AACAGGTCTT CCTTATCTTC  
 301 CCCACTTCTG CTTGGGCCGG GGGCTTATTG ACATGGTGCG GNAACCAGGC  
 351 CATGGCTGAT GCCTTTGANC CCTTGGGAAA AAGGCAGTTC AAGTACCCTG

401 NCTTGGAAGG TGGCGGAAGA ACCTTTTGGC ATGGGAACAG GGCCCCCTTTT  
451 CCTTCTCTTC AACTANTGT TCAAGCACCG AAGCCAACCTC NTGCCACAAG  
501 CCCAGGTAAG GTCTCTGCCA CTCCTGGAGA GAGACGAGGA TGTAGCCCGT  
551 GAACGGGAGC GGGTGGTCCA AGGAGCCACC CAGGGGGATG TGTTGGTGCT  
601 GAGGAACCTG ACCAAGGTAT ACCGTGGGCA GAGGATGCCA GCTGTGACC  
651 GCTTGTGCCT GGGGATTCCC CCTGGTGAGT GTTTTGGGCT GCTGGGTGTG  
701 AACGGAGCAG GGAAGACGTC CACGTTTCGC ATGGTGACGG GGGACACATT  
751 GGCCAGCAGG GCGGAGGCTG TGCTGGCAGG CCACAGCGGG CCCGGGAACC  
801 CAGTGTGCGC ACCTCNAGGG CAGGCNCAGC GTGGCCCCGGG AACCAGTGC  
851 TGCGCACCTA AGCATGGGAT ACTGCCCTNA ATCCGATGCC ATCTTTGAGC  
901 TGCTGACGGG CCGCGAGCAC CTGGAGCTGC TTGCGCGCCT GCGCGGTGTC  
951 CCGGAGGCCC AGGTTGCCCA NACCGNTGGC TCGGGCCTGG CGCGTCTGGG  
1001 ACTCTCATGG TACGCAGACC GGCCTGCAGG CACCTACAGG AACCTGCCCCG  
1051 GGCGGGCCGCT CGAGCCCNNTA NNTGAAGTA 3'

Figure 4b

...CTCCTGCCAC AGTTAGTGAG GTCTATGGAG AGGGTGGCAG GGGCCAAGGA  
CCTACTTTAA GCCACAGAT ATTCTGTCCC CAGGCCAGG GTGAGGTCTC...

Figure 5

CDNA-sequences of lipid sensitive Genes:

ABCB9, ABCA6, ABCC4, ABCA1, ABCD2, ABCB1, ABCB4, ABCC2, ABCD1, ABCC1,  
ABCB6, ABCB11, ABCG2, ABCC5, ABCA5, ABCG1, ABCA3

ABCB9 GENBANK:U66676

GCCAATGNCACGGTTTCATCATGGAAGTCCAGGACGGCTACAGCACAGAGACAGGGGAGA  
AGGGCGCCCAAGCTGTCAGGTGGCCAGAAGCAGCGGGTGGCCATGGCCGNGGCTCTGGTGC  
GGAAACCCCAAGTCTCTCATCTGGATGAAGCCACCAGCGCTTTGGATGCCGAGAGCGAGT  
ATCTGATCCAGCAGGCCATCCATGGCAACCTGTGAGAAGCACACGGTACTCATATCGCG  
CACCGGCTGAGCACCGTGGAGCACGCGCACCTCATTGTGGTGTGGACAAGGGCCGCTA  
GTGACGACAGGGCACCCACCAGCAGCTTGCTTGCCCCAGGGCGGGCTTTTACGGCAAGCTN  
GTTGCAGCGGCAGATGTGGGGTTTCAAGGCGCAGACTTCACAGCTGGCCACAACGAGCC  
TGTAAGCAACGGGTCAAGAGCCGTGATGGGGGGCCCTCCTTCGCCCCGGTGGCAGAGGAC  
CCGGTGCCTGCCTGGCAGATGTGCCACGGAGGTTTCCAGCTGCCCTACCGAGCCAGGC  
CTGCAGCACTGAAAGACGACCTGCCATGTCCCATGATCACCGCTTNTGCAATCTTGGCCC  
TGGTCCCTGCCCCATTCCAGGGCACTCTTACCCCNNTGSGGGGATGTCCAAGAGCATA  
GTCTCTCCCCATACCCCTCCAGAGAAGGGGCTTCCCTGTCCGGAGGGAGACACGGGGAA  
CGGGATTTTCCGTCTCTCCCTCTTGCCAGCTCTGTGAGTCTGGCCAGGGCGGGTAGGGAG  
CGTGAGGGGCATCTGTCTGCCAATTGCCCCGTGCCAATCTAAGCCAGTCTCACTGTGACC  
ACAGGAAACCTCAACTGGGGGAGTGAGGAGCTGGCCAGGTCTGGAGGGGCCTCAGGTGCC  
CCCAGCCCCGACCCAGCTTTGCCCCCTCGTCAATCAACCCCTGGCTGGCAGCCGCCCTC  
CCCACACCCGCCCCCTGTGCTCTGTGCTGTGGAGGCCAGTGGACCTTCATGAGATGCATT  
CTCTTCTGTCTTTGGTGGANGGGATGGTGCAAAGCCCAGGATCTGGCTTTGCCAGAGGTT  
GCAACATGTTGAGAGAAACCGGTCAATAAAGTGACTACCTCTTACCCCT

ABCA6 GENBANK:U66680

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ABCA1 Acc. Nr.: AJ012376 GENBANK: HSA012376

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ABCB4 Acc. Nr.: M23234 GENBANK:HUMMDR3

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ABCA5 Acc.Nr.: AF000148 GENBANK: HSAF000148

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ABCG1 Acc.Nr.: U34919 GENBANK:HSU34919

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ABCA3 Acc.Nr: U78735 GENBANK:HSU78735

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## Fragment 640918

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121 GTTAAACAGAGTTTCGACCTGGAGGAGTACAGCCTCTCACAGTCTACCCCTGGAGCAGGTT  
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241 GTGAAGTGGAACCTCCTCTGCAGGAAGAGCCTTAAAGCTCCAAATACCCCTATATCTTTC  
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361 GGTGGTACAAAATGCATTTGAACTCATGCAATAATTATC

## Fragment 698739

1 GCTCTCCACACAGAGATTTTGAAGCTTTTCCACAGGCTGCTTGGCAGGAAGATATTCC  
61 TCTTTAATGGCGTATAAGTTACCTGTGGAGGATGTCCACCCCTCTATCTCGGGCCTTTTTTC  
121 AAGTTAGAGGCGATGAAACAGACCTTCAACCTGGAGGAATACAGCCTCTCTCAGGCTACC  
181 TTGGAGCAGGTATTCTTAGAACTCTGTAAAGAGCAGGAGCTGGGAAATGTTGATGATAAA  
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361 TCCTTTTACTTCAGTTAACAAAAGAAAACATTTAATAAACATTCAATAATGATTACAGTT  
421 TTCATTTTAAAAATTTAGGATGAAGGAAACAAGGAAATATAGGGAAAAGTAGTAGCAA  
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## Fragment 990006

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## Fragment 1133530

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## Fragment 1125168

## CTGGATT

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## Fragment 1203215

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251 AATCTGTTTC CCGTGCTGCG AGCTGTCAGC A

## Huwhite2

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## SEQUENCE LISTING

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&lt;130&gt; ATP binding cassette genes and protein

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Gly Lys Ile Leu Tyr Thr Pro Asp Thr Pro Ala Thr Arg Gln Val Met  
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Gln Ala Ile Arg Thr Ile Ser Arg Phe Met Glu Cys Val Asn Leu Asn  
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Gln Met Pro Tyr Pro Cys Tyr Val Asp Asp Ile Phe Leu Arg Val Met  
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Ser Arg Ser Met Pro Leu Phe Met Thr Leu Ala Trp Ile Tyr Ser Val  
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Ala Val Ile Ile Lys Gly Ile Val Tyr Glu Lys Glu Ala Arg Leu Lys  
595 600 605

Glu Thr Met Arg Ile Met Gly Leu Asp Asn Ser Ile Leu Trp Phe Ser  
610 615 620

Trp Phe Ile Ser Ser Leu Ile Pro Leu Leu Val Ser Ala Gly Leu Leu  
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Val Val Ile Leu Lys Leu Gly Asn Leu Leu Pro Tyr Ser Asp Pro Ser  
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Val Val Phe Val Phe Leu Ser Val Phe Ala Val Val Thr Ile Leu Gln  
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Cys Phe Leu Ile Ser Thr Leu Phe Ser Arg Ala Asn Leu Ala Ala Ala  
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Cys Gly Gly Ile Ile Tyr Phe Thr Leu Tyr Leu Pro Tyr Val Leu Cys  
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Val Ala Trp Gln Asp Tyr Val Gly Phe Thr Leu Lys Ile Phe Ala Ser  
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Leu Leu Ser Pro Val Ala Phe Gly Phe Gly Cys Glu Tyr Phe Ala Leu  
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Phe Glu Glu Gln Gly Ile Gly Val Gln Trp Asp Asn Leu Phe Glu Ser  
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Pro Val Glu Glu Asp Gly Phe Asn Leu Thr Thr Ser Val Ser Met Met  
755 760 765

Leu Phe Asp Thr Phe Leu Tyr Gly Val Met Thr Trp Tyr Ile Glu Ala  
770 775 780

Val Phe Pro Gly Gln Tyr Gly Ile Pro Arg Pro Trp Tyr Phe Pro Cys  
785 790 795 800

Thr Lys Ser Tyr Trp Phe Gly Glu Glu Ser Asp Glu Lys Ser His Pro  
805 810 815

Gly Ser Asn Gln Lys Arg Ile Ser Glu Ile Cys Met Glu Glu Glu Pro  
820 825 830

Thr His Leu Lys Leu Gly Val Ser Ile Gln Asn Leu Val Lys Val Tyr  
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Arg Asp Gly Met Lys Val Ala Val Asp Gly Leu Ala Leu Asn Phe Tyr  
850 855 860

Glu Gly Gln Ile Thr Ser Phe Leu Gly His Asn Gly Ala Gly Lys Thr  
865 870 875 880

Thr Thr Met Ser Ile Leu Thr Gly Leu Phe Pro Pro Thr Ser Gly Thr  
885 890 895

Ala Tyr Ile Leu Gly Lys Asp Ile Arg Ser Glu Met Ser Thr Ile Arg  
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Gln Asn Leu Gly Val Cys Pro Gln His Asn Val Leu Phe Asp Met Leu  
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Thr Val Glu Glu His Ile Trp Phe Tyr Ala Arg Leu Lys Gly Leu Ser  
930 935 940

Glu Lys His Val Lys Ala Glu Met Glu Gln Met Ala Leu Asp Val Gly

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Met Gln Arg Lys Leu Ser Val Ala Leu Ala Phe Val Gly Gly Ser Lys  
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Val Val Ile Leu Asp Glu Pro Thr Ala Gly Val Asp Pro Tyr Ser Arg  
                         995                      1000                      1005  
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Ile Ala Ile Ile Ser His Gly Lys Leu Cys Cys Val Gly Ser Ser Leu  
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Phe Leu Lys Asn Gln Leu Gly Thr Gly Tyr Tyr Leu Thr Leu Val Lys  
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Lys Asp Val Glu Ser Ser Leu Ser Ser Cys Arg Asn Ser Ser Ser Thr  
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Ala Gly Leu Gly Ser Asp His Glu Ser Asp Thr Leu Thr Ile Asp Val  
1105                      1110                      1115                      1120  
Ser Ala Ile Ser Asn Leu Ile Arg Lys His Val Ser Glu Ala Arg Leu  
                         1125                      1130                      1135  
Val Glu Asp Ile Gly His Glu Leu Thr Tyr Val Leu Pro Tyr Glu Ala  
                         1140                      1145                      1150



Ala Lys Glu Gly Ala Phe Val Glu Leu Phe His Glu Ile Asp Asp Arg  
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Leu Ser Asp Leu Gly Ile Ser Ser Tyr Gly Ile Ser Glu Thr Thr Leu  
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Glu Glu Ile Phe Leu Lys Val Ala Glu Glu Ser Gly Val Asp Ala Glu  
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Thr Ser Asp Gly Thr Leu Pro Ala Arg Arg Asn Arg Arg Ala Phe Gly  
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Asp Lys Gln Ser Cys Leu Arg Pro Phe Thr Glu Asp Asp Ala Ala Asp  
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Pro Asn Asp Ser Asp Ile Asp Pro Glu Ser Arg Glu Thr Asp Leu Leu  
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Ser Gly Met Asp Gly Lys Gly Ser Tyr Gln Val Lys Gly Trp Lys Leu  
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Thr Gln Gln Gln Phe Val Ala Leu Leu Trp Lys Arg Leu Leu Ile Ala  
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Arg Arg Ser Arg Lys Gly Phe Phe Ala Gln Ile Val Leu Pro Ala Val  
1285 1290 1295

Phe Val Cys Ile Ala Leu Val Phe Ser Leu Ile Val Pro Pro Phe Gly  
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Lys Tyr Pro Ser Leu Glu Leu Gln Pro Trp Met Tyr Asn Glu Gln Tyr  
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Thr Phe Val Ser Asn Asp Ala Pro Glu Asp Thr Gly Thr Leu Glu Leu  
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Glu Leu Phe Thr Asp Asn Lys Leu Asn Asn Ile Asn Asp Ile Leu Lys  
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Ser Val Phe Leu Ile Phe Pro His Phe Cys Leu Gly Arg Gly Leu Ile  
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Asp Met Val Lys Asn Gln Ala Met Ala Asp Ala Leu Glu Arg Phe Gly  
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Glu Asn Arg Phe Val Ser Pro Leu Ser Trp Asp Leu Val Gly Arg Asn  
1780 1785 1790

Leu Phe Ala Met Ala Val Glu Gly Val Val Phe Phe Leu Ile Thr Val  
1795 1800 1805

Leu Ile Gln Tyr Arg Phe Phe Ile Arg Pro Arg Pro Val Asn Ala Lys  
1810 1815 1820

Leu Ser Pro Leu Asn Asp Glu Asp Glu Asp Val Arg Arg Glu Arg Gln  
1825 1830 1835 1840

Arg Ile Leu Asp Gly Gly Gly Gln Asn Asp Ile Leu Glu Ile Lys Glu  
1845 1850 1855

Leu Thr Lys Ile Tyr Arg Arg Lys Arg Lys Pro Ala Val Asp Arg Ile  
1860 1865 1870

Cys Val Gly Ile Pro Pro Gly Glu Cys Phe Gly Leu Leu Gly Val Asn  
1875 1880 1885

Gly Ala Gly Lys Ser Ser Thr Phe Lys Met Leu Thr Gly Asp Thr Thr  
1890 1895 1900

Val Thr Arg Gly Asp Ala Phe Leu Asn Arg Asn Ser Ile Leu Ser Asn  
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Ile His Glu Val His Gln Asn Met Gly Tyr Cys Pro Gln Phe Asp Ala  
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2005 2010 2015

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Arg Asn Met Leu Gln Tyr Gln Leu Pro Ser Ser Leu Ser Ser Leu Ala  
2115 2120 2125

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&lt;212&gt; DNA

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1304

&lt;210&gt; 5

&lt;211&gt; 65

&lt;212&gt; PRT

&lt;213&gt; Human

&lt;220&gt;

&lt;223&gt; Partial peptide sequence of ABCG1 (ABC8)

&lt;400&gt; 5

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1

5

10

15

Ser Tyr Val Arg Tyr Gly Phe Glu Gly Val Ile Leu Ser Ile Tyr Gly

20

25

30

Leu Asp Arg Glu Asp Leu His Cys Asp Ile Asp Glu Thr Cys His Phe

35

40

45

Gln Lys Ser Glu Ala Ile Leu Arg Glu Leu Asp Val Glu Asn Ala Lys

50

55

60

Leu

65

&lt;210&gt; 6

&lt;211&gt; 4864

&lt;212&gt; DNA

&lt;213&gt; Human

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&lt;223&gt; human cDNA of ABCC2 (MRP2)

&lt;400&gt; 6

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<220>

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<220>

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<220>

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<400> 11

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&lt;213&gt; Human

&lt;220&gt;

&lt;223&gt; human cDNA of ABCB6

&lt;400&gt; 12

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&lt;211&gt; 4776

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&lt;213&gt; Human

&lt;220&gt;

&lt;223&gt; human cDNA of ABCB11

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&lt;211&gt; 5838

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&lt;213&gt; Human

&lt;220&gt;

&lt;223&gt; human cDNA of ABCC5 (MRP5)

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<213> Human

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<223> human cDNA

<400> 17

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<212> DNA

<213> Human

<220>

<223> human cDNA

<400> 18

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<213> Human

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<223> human cDNA of ABCC4 (MRP4)



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&lt;210&gt; 20

&lt;211&gt; 2911

&lt;212&gt; DNA

&lt;213&gt; Human

&lt;220&gt;

&lt;223&gt; human cDNA of ABCA8 (ABC-new)

&lt;400&gt; 20

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<223> human Intron-Sequence of ABCA8 (ABC-new)

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<400> 22

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<223> human cDNA

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<223> human cDNA

<400> 24

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<223> human cDNA of Huwhite2

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&lt;211&gt; 820

&lt;212&gt; DNA

&lt;213&gt; Human

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&lt;223&gt; human cDNA

&lt;400&gt; 26

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&lt;210&gt; 27

&lt;211&gt; 575

&lt;212&gt; DNA

&lt;213&gt; Human

&lt;220&gt;

&lt;223&gt; human cDNA

&lt;400&gt; 27

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&lt;210&gt; 28

&lt;211&gt; 300

&lt;212&gt; DNA

&lt;213&gt; Human

&lt;220&gt;

&lt;223&gt; human cDNA

&lt;400&gt; 28

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&lt;212&gt; DNA

&lt;213&gt; Human

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&lt;400&gt; 29

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&lt;210&gt; 30

&lt;211&gt; 6491

&lt;212&gt; DNA

&lt;213&gt; Human

&lt;220&gt;



<223> human cDNA of ABCA3 (ABC3)

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6491

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&lt;211&gt; 2923

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2923

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&lt;211&gt; 13

&lt;212&gt; DNA

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5

10

15

&lt;210&gt; 54

&lt;211&gt; 2923

&lt;212&gt; DNA

&lt;213&gt; Human

&lt;220&gt;

&lt;223&gt; human genomic DNA of 5'-UTR of ABCG1

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May 16

1 silver }  
1 gold }

427 4500064

116.46

03942

544 491327

427413448

gold + sage

174 -548

42.95

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